6-2018

Microgravity Exerts an Age-Dependent Effect on Cardiovascular Progenitor Cell Development

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School of Medicine
in conjunction with the
Faculty of Graduate Studies

Microgravity Exerts an Age-Dependent Effect on Cardiovascular Progenitor Cell Development

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Anatomy

June 2018

Each person whose signature appears below certifies that this dissertation in their opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ACKNOWLEDGEMENTS

That relating the role of my friends, family members, and colleagues in my growth as both a person and scientist, without diluting the depth of my gratitude, should be possible, I could only begin by acknowledging their profound influence over my intellectual and emotional development. Yet, I would be remiss not to acknowledge those whose time, sacrifices, and contributions are explicitly tied to my doctoral training.

Thus, I first thank my mentor, Mary Kearns-Jonker, for providing a thorough scientific training, characterized by her desire to ensure that I always "do right by my experiments and right by people." Her genuine regard for my well-being and belief in my ability to learn are reflected by both my happiness and this dissertation.

I am indebted to my dissertation committee members for their thoughtful feedback and challenging interactions. In particular, I thank Christopher G Wilson for his patience and enthusiasm while I learned to perform electrophysiology experiments. Similarly, I thank the staff of BioServe Space Technologies for their support in making launching our International Space Station experiments a more pleasant experience. I also thank the Anatomy Department for their support and CASIS (GA-2014-130 to MKJ) for funding many of the experiments described throughout this dissertation.

I thank my father for his perennial support of my education and unconditional love. Much of who I am and what I have accomplished is thanks to him. Finally, I am grateful for my loving husband, Ben. While I could cite his support, patience, and encouragement as being foundational to my successes in graduate school, his qualities as a human being and as a partner extend far beyond these immediate contributions and can be found in my renewed sense of gratefulness and excitement for life.

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ABBREVIATIONS

AGTR1/2	Angiotensin II Receptor Type 1/2
AKT	Protein Kinase B
AP	Action Potential
APD50	Action potential duration at 50% of the maximum amplitude
bFGF	Basic Fibroblast Growth Factor
BMC	Bone Marrow-derived Cells
CADUCEUS	CArdiosphere-Derived aUtologous StemCElls to reverse ventricUlar dysfunction
CAMK2A	Calcium/Calmodulin-Dependent Protein Kinase II Alpha
CD117 (or CKIT)	Cluster of Differentiation 117
CD140A (or PDGFRA)	Cluster of Differentiation 140A
CDC42	Cell Division Control Protein 42
cDNA	Complementary Deoxyribonucleic Acid
CKIT (or CD117)	Tyrosine Protein Kinase Kit
CM	Cardiomyocyte
CPC	Cardiovascular Progenitor Cells
CTNNB1	Catenin Beta 1
CX43 (or GJA1)	Connexin 43
CX45 (or GJC1)	Connexin 45
CXCR4	Chemokine (C-X-C Motif) Receptor 4
E2F1	E2 Transcription Factor 1

EF	Ejection Fraction
EGFR	Epidermal Growth Factor Receptor
ELK1	ETS Transcription Factor
EOMES	Eomesodermin
ERK	Extracellular Signal-Regulated Kinase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GATA4	GATA Binding Protein 4
GJA1 (or CX43)	Gap Junction Protein Alpha 1
GJC1 (or CX45)	Gap Junction Protein Gamma 1
GRB2	Growth Factor Receptor Bound Protein 2
GSC	Goosecoid
GSK3B	Glycogen Synthase Kinase 3 Beta
HCN4	Hyperpolarization-Activated, Cyclic Nucleotide-Gated Channel 4
hESC	Human Embryonic Stem Cells
HGF	Hepatocyte Growth Factor
IGF-1	Insulin-Like Growth Factor 1
IGF-1R	Insulin-Like Growth Factor 1 Receptor
IGF-2	Insulin-Like Growth Factor 2
iPSC	Induced Pluripotent Stem Cells
ISL1	Islet-1
ISS	International Space Station
KDR (or VEGFR2)	Kinase Insert Domain Receptor

LVEF	Left Ventricular Ejection Fraction
MAPK	Mitogen-Activated Protein Kinase
MEIS1	Meis Homeobox 1
MESP1	Mesoderm Posterior BHLH Transcription Factor 1
MESP1	Mesoderm Posterior Protein 1
MG	Microgravity
miRNA	MicroRNA
MLC2V	Myosin Regulatory Light Chain 2 Ventricular
MSC	Mesenchymal Stem Cell
NFATC3	Nuclear Factor of Activated T Cells, Cytoplasmic 3
NFKB1	Nuclear Factor Kappa B Subunit 1
NKX2-5	NK2 Homeobox 5
OCT4	Octamer-Binding Protein 4
pAKT	Phosphorylated Protein Kinase B
PDGF	Platelet-Derived Growth Factor
PDGFRA (or CD140A)	Platelet-Derived Growth Factor Receptor Alpha
pERK	Phosphorylated Extracellular Signal-Regulated Kinase
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3’ Kinase
PLCG1	Phospholipase C gamma 1
PLK1	Polo-like Kinase 1
pPKC	Phosphorylated Protein Kinase C Alpha
PRKCA	Protein Kinase C Alpha

RAC1	Ras-related C3 botulinum toxin substrate 1
RAD23	DNA Repair Protein RAD23
RAD50	DNA Repair Protein RAD50
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
RHOA	Ras Homolog Family Member A
RNA	Ribonucleic Acid
RT-PCR	Real-Time Polymerase Chain Reaction
RUNX2	Runt-Related Transcription Factor 2
SAN	Sinoatrial Node
SCA-1	Stem Cell Antigen-1
SCIPIO	Stem Cell Infusion in Patients with Ischemic Cardiomyopathy
SDF-1α	Stromal Cell-Derived Factor 1α
SHOX2	Short Stature Homeobox 2
SIRPα	signal-regulatory protein alpha
SMG	Simulated Microgravity
SSEA-1	Stage Specific Embryonic Antigen 1
T	T-Box T (or Brachyury)
TAC-HFT	Transendocardial Mesenchymal Stem Cells and Mononuclear Bone Marrow Cells for Ischemic Cardiomyopathy Trial
TBX1	T-Box 1
TBX3	T-Box 3

TBX5	T-Box 5
TBX18	T-Box 18
TBX20	T-Box 20
TGFB1	Transforming Growth Factor, Beta 1
TIME	Effect of the Use and Timing of Bone Marrow Mononuclear Cell Delivery on Left Ventricular Function After Acute Myocardial Infarction
TNNT2	Troponin T
VEFGA	Vascular Endothelial Growth Factor A
VEGFR1	Vascular Endothelial Growth Factor Receptor 1
VEGFR2 (or KDR)	Vascular Endothelial Growth Factor Receptor 2
vWF	Von Willebrand Factor
WNT2B	Wnt Family Member 2B
WNT3A	Wnt Family Member 3A
WNT5A	Wnt Family Member 5A
WNT9A	Wnt Family Member 9A

ABSTRACT OF THE DISSERTATION

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Cardiovascular Progenitor Cell Development

by

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Loma Linda University, June 2018
Dr. Mary Kearns-Jonker, Chairperson

The heart and its cellular components are profoundly altered by missions to space and injury on Earth. Increasing evidence has identified that one such alteration induced by spaceflight is the promotion of the efficacious use of stem cells in therapies on Earth. For this reason, neonatal and adult human cardiovascular progenitor cells (CPCs) were cultured aboard the International Space Station (ISS). Subsequently, we assessed the effects of mechanical unloading on developmental properties and signaling. Spaceflight induced the expression of genes that are typically associated with an earlier state of cardiovascular development. In particular, in neonatal CPCs, we measured increased expression of pre-cardiac and developmental regulatory (Bmp and Tbx) genes; decreased expression of mesodermal derivative markers, including endothelial tube formation; and enhanced proliferative potential, as indicated by cell growth and cell cycle analysis. Interestingly, these changes were not observed in adult CPCs. To understand the mechanism by which such changes occurred in neonatal CPCs, we assessed the expression of mechanosensitive small RhoGTPases. Given the effect of these molecules on intracellular calcium levels, we evaluated changes in non-canonical Wnt/calcium signaling. ISS-cultured CPCs exhibited elevated levels of calcium handling and signaling

genes, which corresponded to activation of protein kinase C alpha (PKCα), a calcium-dependent protein kinase, and Akt, a regulator of stem cell self-renewal, after 30 days. To explore the effect of calcium induction in neonatal CPCs, we activated PKCα using hWnt5a treatment on Earth, which resulted in an induction of early cardiovascular developmental marker expression. Interestingly, markers of the sinoatrial node, which may represent embryonic myocardium maintained in its primitive state, were induced by culture of neonatal CPCs aboard the ISS, which was modeled on Earth, at least in part, using the calcium signaling activators angiotensin II and hWnt5a. To test whether such signaling could induce sinoatrial nodal gene development on Earth, we treated neonatal CPCs with angiotensin II and observed the reliable induction of a sinoatrial nodal phenotype. We found that, in neonatal CPCs, spaceflight induces PKCα and Akt signaling, promotes the induction of an earlier developmental state, and highlights signaling events that may underpin biological pacemaker development on Earth.

CHAPTER ONE

INTRODUCTION

Heart failure impacts 2% of adults in developed countries (McMurray, 2005) and results in death within one year in 35% of patients (National Clinical Guideline Centre, 2010). There is no cure for heart failure and so treatment relies upon symptom management and the prevention of disease progression. Heart failure can arise due to myocardial infarction, in which the working myocardium dies following artery blockage and reduced access to nutrients and oxygen. Since the human heart lacks a significant natural capacity to regenerate injured or damaged heart muscle (Kikuchi et al., 2012), injuries to cardiac tissue often significantly impact a person's quality of life. In an effort to identify methods to repair damaged myocardium, the transplantation of cardiovascular progenitor cells (CPCs), which retain the ability to differentiate into heart muscle tissue, has been the subject of intensive research. As these studies have evolved, their use in engineering other cardiovascular tissue, such as a biological pacemaker, has progressed as well. Ultimately, research into the biology of CPCs will yield important insights into novel treatments for cardiac repair.

Progenitor Cells for Cardiovascular Repair

Cardiovascular progenitor cells represent a population of multipotent stem cells that reside in the heart through adulthood and exhibit an ability to differentiate into various cardiovascular lineages. CPCs can be derived from primary sources, including the human heart, or can be induced via small molecule-mediated differentiation of

pluripotent or embryonic stem cells, with several markers having been identified on these cells (Table 1).

Cardiovascular progenitor biology is intimately related to the molecular processes that guide the development of cardiac tissue. Yet, given the complexity of cardiovascular development, there are several markers that can be used to identify various cardiovascular precursor cells *in vitro*, as shown in Table 1. In these studies, various methods have been used for the selection of cell markers, including magnetic bead sorting for a particular marker (e.g., PDGFRα in Chong et al. (2013) or c-Kit in Li et al. (2012)). Thus, these reports are limited in their ability to discuss the levels of expression of the identified markers beyond what is detectable using their specified system of isolation. Accordingly, it is possible that such studies excluded cell types that expressed these markers at lower levels. As we discuss later, the overlap in developmental staging and marker expression documented in the studies included in Table 1 may be clarified by a greater understanding of the molecular markers of the various stages of cardiogenesis and the pathways used to drive this process.

Notably, controversy still exists regarding the true constitution of a cardiac progenitor. One laboratory concluded that "resident c-kit(+) cells in the heart are not cardiac stem cells" based upon the inability of c-kit-labeled cells to co-localize with Nkx2.5 or develop into cardiomyocytes that express troponin T (Sultana et al., 2015). Meanwhile, lineage tracing of progenitors that express Isl1, a LIM homeodomain transcription factor, indicated that Isl1-positive cells give rise to the outflow tract, right ventricle, and much of the atria in the developed heart (Cai et al., 2003).

Table 1. Cell types identified or speculated as being cardiovascular progenitors

Progenitor Name	Expression Profile	Reports
Cardiospheres	$CD34^-/45^-$ Central: $c\text{-}kit^+/Nkx2.5^+$ Peripheral: $CD31^+/90^+/105^+/133^+$	Davis et al., 2009
Cardiosphere-derived $c\text{-}kit^+$	$c\text{-}Kit^+/CD29^+/90^+/105^+/45^-/34^-/31^-/133^-$ $c\text{-}kit^+$	Li et al., 2012
	• Vascular: $KDR^+/CD31^+$	Sandstedt et al., 2010
	• Myogenic: $\alpha SA^{+/-}$	Bearzi et al., 2009
$Isl1^+/c\text{-}Kit^+$ Fetal	$Isl1^+/c\text{-}Kit^+$	Serradilfalco et al., 2011
$Isl1^+/c\text{-}Kit^+$ Neonatal & Adult	$Isl1^+/c\text{-}Kit^+/SSEA\text{-}1^+/$ $MESP1^+/PDGFR\alpha^+/$ $KDR^+/CXCR4^+/CXCR7^+$	Fuentes et al., 2013 Baio et al., 2018
$Isl1^+$ hESC-derived	$Isl1^+/Nkx2\text{-}5^+/KDR^+/CD82^+$ $Gata4^+/Tbx1^+/Tbx20^+$	Moretti et al., 2006 Takeda et al., 2018
$Sca\text{-}1^+$	$Sca\text{-}1^+/GATA4^+/NKX2\text{-}5^{+/-}$ $c\text{-}Kit^{+/-}/cd31^-$	Oh et al., 2003 Wang et al., 2006
$SSEA\text{-}1^+$ hESC-derived	$SSEA\text{-}1^+/MESP1^+/Oct4^+$ $SSEA\text{-}1^+/Isl1^+$	Blin et al., 2010[a] Menasché et al., 2015

Abbreviations: CPC, cardiovascular progenitor cells; CXCR4, C-X-C Motif Chemokine Receptor 4; ESC, embryonic stem cells; KDR, kinase-insert domain receptor; MESP1, mesoderm posterior protein 1; PDGFRα, platelet-derived growth factor receptor alpha; SCA-1, stem cell antigen-1; SSEA-1, stage-specific embryonic antigen 1

While both c-Kit and Isl1 are expressed during the process of cardiogenesis, their co-expession with additional markers allow for a more precise definition of the developmental staging of the cell under study. Furthermore, several of the markers defined above may also be helpful in identifying a population of cardiovascular progenitors that exhibit enhanced efficacy in repair, as discussed below. These considerations first require a brief review of the anatomical and molecular events underpinning heart development.

Cardiovascular Development

The Heart Fields

The broad pattern of human heart formation has been well known for decades. Following gastrulation, two, bilateral populations of cranial lateral plate mesoderm develop and fuse at the midline to form the primitive heart tube (Mjaatvedt, 2001). The cardiogenic mesoderm that forms this primitive heart tube is termed the primary heart field. In the early 2000s, a second region of pharyngeal mesoderm was observed to contribute to the elongation of the heart tube. This region, the second heart field, gives rise to tissue that is found at the inflow tract, including parts of the atria and, perhaps, the sinoatrial node (SAN). Notably, the origins of the SAN are still the subject of intensive debate. Indeed, an avian model of cardiovascular development demonstrated that the SAN uniquely arises from the "tertiary heart field," or a mass of mesoderm that is not located within, but rather posterior to, the second heart field (Bressan, 2013).

Following the linear heart tube stage (i.e., prior to heart tube looping), the second heart field also contributes to the outflow tract (Kelly, 2012). Cranial and caudal folding

moves this anteriorly-located cardiogenic plate to the region of the putative thorax. Due to restrictions on growth that are conferred by both the persistence of dorsal mesocardium as well as the developing body walls and diaphragm, the heart tube begins to loop, ultimately forming the inverted, multi-chambered conical shape. Importantly, neural crest cells from the fourth and sixth pharyngeal arches migrate and transform into mesenchyme to contribute to the development and septation of the outflow tract of the heart (Bradshaw, 2009). Therefore, in addition to the tissue of the mesoderm-derived primitive heart tube and of the second heart field, ectoderm-derived, neural crest tissue also contributes to heart formation (Sadler, 2012).

Throughout this process, nearby tissues secrete morphogens, thereby establishing gradients that modulate heart formation. Briefly, lateral ectoderm and endoderm secrete FGFs and BMPs that support cardiac growth ventrally while neuroectoderm-derived WNT inhibits cardiac growth dorsally (Bruneau, 2012). This process of gross, anatomical cardiogenesis is driven by an underlying cascade of molecular events.

Molecular Cardiogenesis

This gradient of morphogens is intimately involved in the molecular events that drive heart formation. An abbreviated version of the molecular events driving cardiogenesis from a pluripotent state is shown in Figure 1 (Gessert & Kühl, 2010; den Hartogh et al., 2015). Progenitor cells used in cardiac stem cell therapies (Table 1) reflect a particular stage of development and thus are limited in their potential applications. As discussed below, consideration of the developmental staging of the progenitor used for transplantation is related to the efficacy of treatment. The schematic shown herein is a

simplified rendering of the events of embryogenesis and include exogenous reagents used in the laboratory to guide these processes for the specific purpose of generative cardiovascular derivatives. For example, Activin A ligand does not have a defined role in mouse embryogenesis (Matzuk et al., 1995), but its signaling pathway is active during early gastrulation (Cheng et al., 2003). Thus, our rendering of cardiogenesis simply serves to both situate the myriad of cardiovascular progenitor cells used for therapies in a developmental context and highlight the potential of each stage to produce various cardiovascular derivatives. Although a thorough discussion of the topic of stem cell plasticity at each stage of development is beyond the scope of this dissertation, the importance of a sufficiently early developmental state in cardiac repair has been highlighted in clinical trials and case reports, as discussed below.

Cardiovascular Progenitor Cell Uses in Regenerative Medicine

The use of a cardiac progenitor that has direct cardiomyogenic potential, such as endogenous CPCs, during transplantation may facilitate regeneration. Current clinical trials involving cardiovascular progenitors to stimulate repair in damaged heart tissue are promising (Bolli, 2011; Makkar, 2012; Gerbin, 2015); however, they are stymied by a failure of cells to engraft into the host tissue and by the use of progenitor types that are restricted in potency (Hong, 2014). Therefore, identifying the molecular events that promote enhanced stemness and regenerative potential in CPCs will benefit stem cell-

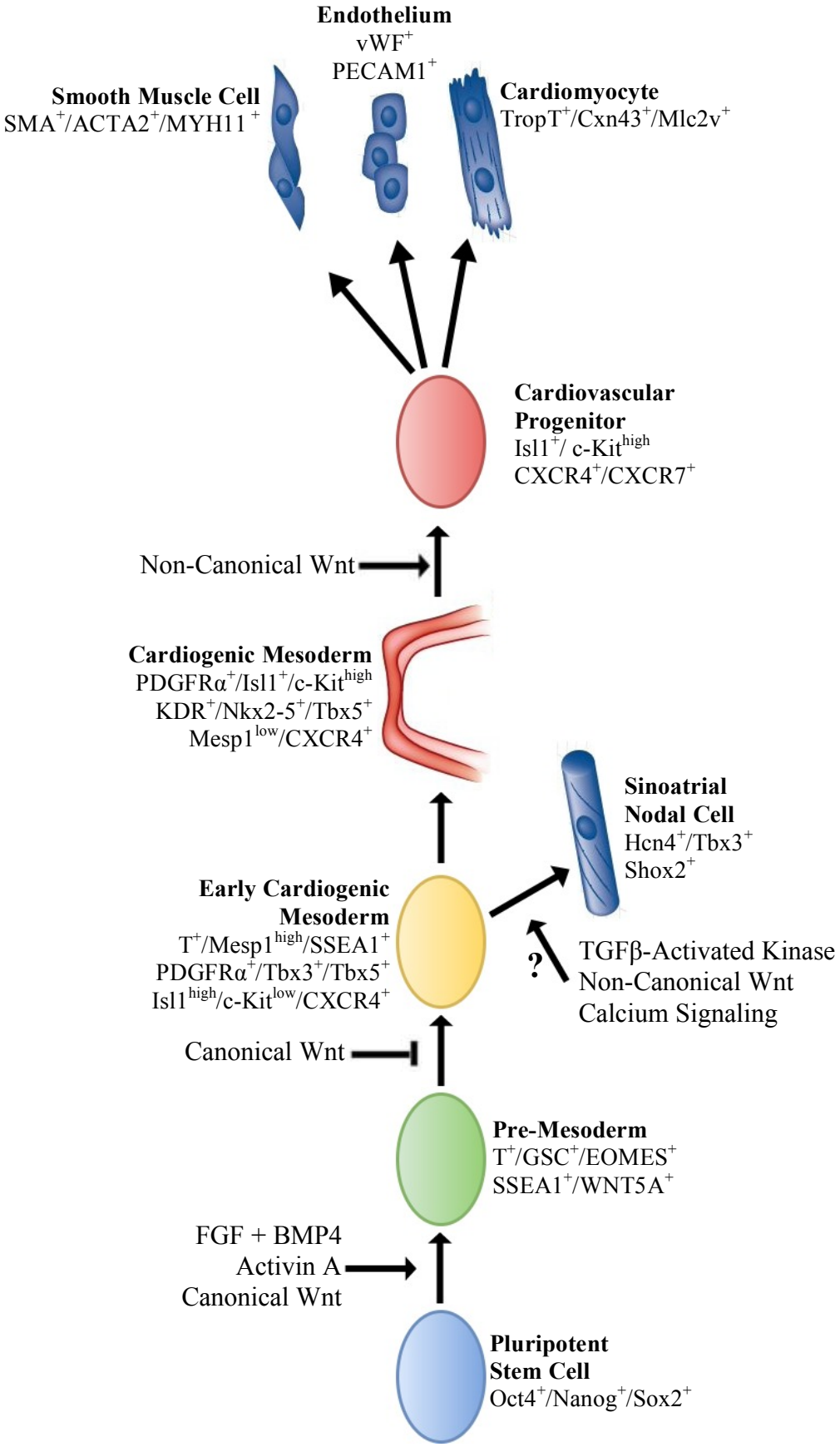
Endothelium
vWF+
PECAM1+
Smooth Muscle Cell
SMA+/ACTA2+/MYH11+
Cardiomyocyte
TropT+/Cxn43+/Mlc2v+
Cardiovascular Progenitor
Isl1+/ c-Kithigh
CXCR4+/CXCR7+
Non-Canonical Wnt
Cardiogenic Mesoderm
PDGFRα+/Isl1+/c-Kithigh
KDR+/Nkx2-5+/Tbx5+
Mesp1low/CXCR4+
Sinoatrial Nodal Cell
Hcn4+/Tbx3+
Shox2+
Early Cardiogenic Mesoderm
T+/Mesp1high/SSEA1+
PDGFRα+/Tbx3+/Tbx5+
Isl1high/c-Kitlow/CXCR4+
?
TGFβ-Activated Kinase
Non-Canonical Wnt
Calcium Signaling
Canonical Wnt
Pre-Mesoderm
T+/GSC+/EOMES+
SSEA1+/WNT5A+
FGF + BMP4
Activin A
Canonical Wnt
Pluripotent Stem Cell
Oct4+/Nanog+/Sox2+

Figure 1. Molecular cardiogenesis is guided by spatiotemporally regulated morphogens and transcription factors

Morphogens and extracellular contact-mediated inductive cues activate signaling pathways that induce mesoderm development, subsequent commitment to the cardiac lineage, and the ultimate development of definitive cardiovascular derivatives. These pathways have been manipulated *in vitro* to culture cardiomyocytes from induced pluripotent, embryonic, and cardiovascular progenitor cells. Although several markers have been identified, the precise temporal regulation of each of these markers remains largely unknown.

based cardiac repair. Additionally, the successful use of autologous CPC sources to regenerate heart tissue in a predominately adult population requires additional understanding of the differences between adult and neonatal CPCs. Our laboratory has previously documented differences in age-dependent expression of microRNAs related to MAPK signaling, cytoskeleton regulation, adherens junction expression, and focal adhesion maintenance (Fuentes, 2013). As will be discussed later, several of these processes are affected by spaceflight. Moreover, while the functional shortcomings of adult CPCs require further elucidation, they are not the only barrier to effective cardiac regeneration based upon autologous cell therapy.

Identifying the appropriate progenitor type that can be used in transplantation is still the subject of debate that has immense implications for cardiovascular repair. Previous studies that employed hematopoietic stem cells (Murry, 2004; Balsam, 2004) and mesenchymal stem cells (MSCs; Dixon, 2009) raised concerns over the myogenic capacity of these cell types. Godier-Furnemont, et al. (2011) employed MSCs in one such study. While enhanced angiogenic potential was noted, the authors acknowledged that cardiomyogenic differentiation of MSCs was unlikely. Employing these progenitor types that are not closely related to cardiac derivatives have resulted in underwhelming clinical trials (Segers, 2008). Since cardiomyocyte proliferation is limited, it has been challenging to transplant myocytes and achieve tissue-like cell densities (Sreejit, 2013; Dubois, 2010; Beitnes, 2009). In addition to cell lineage, research into the developmental status of the stage has provided encouraging results both in large animal models and human cases.

Interestingly, recent evidence has emerged that a progenitor cell that represents an early cardiovascular progenitor may facilitate myocardial repair. In a study of repair

Table 2. Animal studies, case reports, and clinical trials of stem cell-based cardiovascular repair

Cell source	Dev Stage	Outcome (vs Control)	Study
$KDR^+/PDGFR\alpha^+$ hESC-derived CPCs	Early	+28% FS (at 28 days)	Rodent (Fernandes et al., 2015)
$SSEA\text{-}1^+/MESP1^+/Oct4^+$ hESC-derived CPCs	Early	-20% Scar Size (at 2 months)	Non-Human Primate (Blin et al., 2010)
$SSEA\text{-}1^+/Isl1^+$ hESC-derived CPCs	Early	+10% LVEF (at 3 months)	Case Report in Human (Menasché et al., 2015)
$c\text{-}Kit^+$ cardiospheres	Late	Unchanged LVEF (at 1 year)	Human: CADUCEUS (Malliaras et al., 2014)
		+2% LVEF (at 6 months)	(Makkar et al., 2012)
$c\text{-}Kit^+$ Adult CPCs	Late	+13.7 ± 4.5% LVEF[a] (at 12 months)	Human: SCIPIO (Chugh et al., 2012)
		+7.6 ± 2.4% LVEF[a] (at 4 months)	(Bolli et al., 2011)
MSCs	Late	+8% EF[a] (at 12 months)	Human: POSEIDON (Hare et al., 2017)
		+5% EF[a] (at 6 months)	(Hare et al., 2012)
MSCs	Late	+8% EF[a] (at 12 months)	Human: TRIDENT (Hare et al., 2017)
$c\text{-}Kit^+$ CPCs	Late	+4% FS +8% LVEF (at 35 days)	Rodent (Tang et al., 2010)
$Nkx2\text{-}5^+/SIRP\alpha^{+b}$ iPSC-derived CPCs	Late	+4% LVEF (at 4 weeks)	Rodent (Ja et al., 2015)
$CD34^{low}$ BMC-derived MSCs	Late	Unchanged LVEF/EF (at 30 days)	Human: TAC-HFT (Heldman et al., 2014)
$CD34^{low}/CD133^{low}$ BMCs	Late	-3% LVEF (at 6 months)	Human: LateTIME (Traverse et al., 2011)
BMCs	Late	Unchanged Measures	Human: FOCUS-CCTRN (Perin et al., 2012)

[a]No control group included; [b]In the context of cardiovascular development, signal-regulatory protein alpha (SIRPα) is indicative of a cardiomyocyte population (Dubois, 2011).

following myocardial infarction in nun-human primates, Blin et al. (2010) studied the efficacy of an early-stage cardiovascular progenitor that expressed stage-specific embryonic antigen 1 (SSEA-1), mesoderm posterior 1 (MESP1), and OCT4. The researchers observed differentiation of these early cells into ventricular cardiomyocytes, thereby healing approximately 20% of the scar tissue. The use of such a progenitor was demonstrated to be efficacious in a rodent model of myocardial repair (Bellamy, 2015). Meanwhile, Menasché et al. (2015) reported the first clinical case report of treatment using this early cardiovascular progenitor cell type. In brief, transplantation of SSEA-1- and Islet1-expressing CPCs derived from embryonic stem cells resulted in improved cardiovascular function. As shown in Table 2, studies of stem cell-based cardiovascular repair have revealed an emerging pattern in which an earlier cardiovascular progenitor more effectively improves cardiovascular function.

Ultimately, increasingly strong evidence supports the use of progenitors that are only nascent in their commitment to the cardiovascular lineage in promoting cardiovascular repair. Moreover, recent evidence involving early CPCs that express Mesp1, SSEA-1, and Islet1 suggests that the developmental timing of cells used in such therapies is critical to the regeneration of cardiovascular function (Blin, 2010; Menasché, 2015). Thus, research in cardiovascular regenerative medicine must also consider methods that enhance the regenerative potential of CPCs, possibly through the induction of an early developmental state.

Efforts to identify and produce an earlier developmental state in stem cell populations have largely been studied *in vitro*. Yet, traditional cell culture conditions do not reflect the complex biomechanical processes of embryogenesis *in utero.* One major

difference between two-dimensional *in vitro* culture and the developing embryo is the presence of a buoyancy force generated by amniotic fluid that counteracts the effects of gravity (Andreazzol, 2017). One magnetic resonance imaging-based study estimated that, up until approximately the 21st week of gestation, the embryo and fetus develop in conditions that resemble neutral floating (Sekulic, 2005). Moreover, the human embryo does not undergo skin keratinization (i.e., body wall "hardening") until after approximately gestational week 20 (Underwood, 2005), thus indicating that the buoyancy force generated by the amniotic fluid is transmitted to the fluid inside the embryo. Thus, to the extent that intracellular densities of embryonic cells are of the same density as the amniotic fluid (Valles, 2002), the embryo *in utero* experiences conditions of relative weightlessness, or reduced gravity. For this reason, researchers have turned their attention to recapitulating the physics of intrauterine development more closely. Research by the laboratory of Alan Flake has demonsrated that a biomimetic uterus can effectively facilitate proper development in extremely premature lamb (Partridge, 2017). Elsewhere, three-dimensional cultures and microgravity have been studied to understand how such conditions can promote stem cell development and function.

Stem Cells and Reduced Gravity Conditions

Humankind has long been interested in our expanded presence in space. Although only a handful of astronauts ever spend time in orbit, the technology that is developed by NASA for spaceflight has been broadly shared with society. Such advances have impacted many facets of human society on Earth, including transportation, public safety, health and medicine, and computer technology. With advances in the life science research

capacity of the National Lab aboard the International Space Station (Blaber, 2014), biologists have become empowered to similarly advance stem cell research for the benefit of society both in space and on Earth. In the context of cardiac stem cell therapies, as further discussed below, microgravity increasingly appears to represent one avenue by which a desirable stem cell population can be achieved.

In reviewing the myriad studies regarding stem cells under simulated microgravity or aboard a space-bound vessel, several trends have emerged. First, stem cells exhibit reduced terminal derivative and enhanced self-renewal and stemness marker expression during exposure to reduced gravity conditions (Blaber, 2015; Fuentes, 2015). Second, upon return to normal gravity conditions, microgravity-exposed cells are more readily able to differentiate into neurons (Chen, 2011), bone nodules (Blaber, 2014), or cardiomyocytes (Jha, 2016). As researchers continue to explore the adaptation of stem cells to reduced gravity conditions, the prospect of engineering tissue using microgravity-exposed progenitors has increased. However, a greater understanding of the mechanism by which stem cells adapt to reduced gravity conditions is needed in order to enjoy the benefits of microgravity-cultured stem cells in clinics on Earth.

Mechanotransduction under Microgravity

Across models and culture systems, molecular biologists have increasingly identified the role of small RhoGTPases in mediating the response of cells to a reduced gravity environment (Meyers, 2006; Louis, 2015). RhoA and CDC42 are small RhoGTPases that associate with the actin cytoskeleton (Cenni, 2003), participate in the non-canonical Wnt planar cell polarity pathway (Komiya, 2008), and modulate

intracellular calcium potentiation (Kim, 2009). Other experiments have identified alterations to small RhoGTPases, such as the suppression of RhoA in MSCs, in response to SMG (Seki, 2006). Elsewhere, researchers have reported the involvement of the primary cilium, which coordinates the early molecular events of cardiogenesis (Clement, 2009), as a sensor of cell-cell contact and altered mechanical stress (Moorman, 2007). Interestingly, RhoA activity was found to be related to ciliogensis (Pan, 2007), further supporting the hypothesis that small RhoGTPases have an important role in cardiac development.

Ultimately, this reduction in *RHOA* expression logically follows from the reduced mechanical stress that is expected under microgravity conditions. Furthermore, mechanotransduction-induced changes in RhoA expression would be expected to impact calcium handling. Indeed, substrate rigidity (or the lack thereof in this context), has been shown to directly impact calcium oscillations within MSCs. Using fluorescence resonance energy transfer, Kim et al. (2009) observed changes to Ca^{2+} oscillation in accordance with changes to the stiffness of the MSC culture environment. Similarly, we found that calcium pathway genes and those related to calcium handling were expressed at higher levels under SMG (Baio, 2018). Meanwhile, the modification of signaling pathways related to calcium, either as an important secondary or mediating molecule, directly impacts the ability of CPCs to differentiate or maintain pluripotency (Tonelli, 2012; Apati, 2016). Such findings indicate that research into the molecular events underpinning the adaptation of stem cells to reduced gravity conditions will have implications for regenerative medicine here on Earth.

Potential Therapeutic Benefits of Reduced Gravity

Research in our own laboratory has shown that simulated microgravity (SMG) impacts the developmental profile of human cardiovascular progenitor cells (Fuentes, 2015). In particular, earlier cardiac developmental genes were induced in neonatal CPCs following exposure to SMG for 6-7 days. The observation that a sufficiently early, cardiac-committed progenitor exhibits enhanced efficacy upon transplantation *in vivo* (Bellamy, 2015; Blin, 2010; Menasché, 2015) prompts molecular biologists to further explore the potential therapeutic use of reduced gravity conditions in advancing stem cell therapies. Initial research into the mechanism governing these observations indicated that small RhoGTPases and Wnt signaling as being some of the systems affected by the simulated microgravity environment. Elsewhere, the effect of mechanical unloading on mouse embryonic stem cells (ESCs) has been shown to impact differentiation and stemness, with experiments by Blaber et al. (2015) demonstrating that embryoid bodies retain markers of self-renewal and exhibit reduced definitive germ layer marker expression when flown in space. However, in the same studies, when mechanically unloaded embryoid bodies returned to Earth, they were able to differentiate more readily into contractile cardiomyocyte colonies. Similarly, Jha et al. (Jha, 2016) found that human induced pluripotent stem cells more readily differentiate into cardiomyocytes using three-dimensional culture coupled with transient, early exposure to SMG. These separate experiments may represent a similar phenomenon in which a low gravity culture promotes an enhanced state of stemness under SMG or microgravity (MG) that results in increased differentiation ability when the cells are returned to normal gravity conditions. Thus, stem cell therapies relevant to cardiac repair may be improved by manipulating the

mechanisms relevant to mechanical signaling in cardiovascular progenitors. In particular, inducing enhanced stemness in cardiovascular progenitors may facilitate a correspondingly enhanced clinical effect upon transplantation.

Alterations in mechanical sensing molecules, such as the small RhoGTPases, are believed to be involved in the molecular adaptation to microgravity (MG; Meyers, 2005; Louis, 2015). Importantly, these molecules are also able to impact intracellular signaling pathways, such as calcium oscillations, which subsequently can activate AKT (Gocher, 2014) and extracellular-signal regulated kinase (ERK; Schonwasser, 1998). In the context of cardiogenesis, these processes are critical to maintaining a balance between inductive and proliferative cues (Romorini, 2016; Pucéat, 2005). Therefore, manipulating the normal gravity environment of early CPCs may highlight important mechanisms by which early cardiac progenitors develop or expand. Such insights may be applied to further understand cardiovascular development and to enhance the outcomes of stem cell-based regenerative therapies. Therefore, the application of findings from microgravity experiments to Earth-based experiments may help overcome the shortcomings of current clinical trials involving the use of CPCs for cardiac repair.

Sinoatrial Nodal Induction under Reduced Gravity

Two lines of evidence support the application of reduced gravity-mediated signaling events to sinoatrial nodal development. First, the induction of an earlier developmental state logically supports the development of sinoatrial nodal cells, which are hypothesized to be myocardial cells maintained in an embryonic state (Bakker, 2010). Second, recent studies of CPCs in our laboratory (Baio, 2018) and a recent study of

neural crest progenitors (Hatzistergos, 2018) both demonstrated that simulated microgravity induced the expansion of mesoderm-derived pacemaker sinoatrial nodal cells.

In considering the first line of evidence, several studies have shown that an early developmental state may provide, or simply constitute, the appropriate cell source for biological pacemaker development. In an avian model, Bressan et al. (2013) used fate mapping to determine that sinoatrial nodal cells were observed to already be specified shortly after gastrulation prior to the onset of cardiogenesis within a region that was observed to be Nkx2-5- and Isl1-negative. Meanwhile, the Keller group recently reported a method of generating sinoatrial nodal-like pacemaker cells in Nkx2-5-negative cardiomyocytes (Protze, 2017). In this way, both groups demonstrate that developmental timing is critical to the development of this pacing structure. Finally, studies of definitive sinoatrial nodal cells indicate that primitive myocardium features, such as a simple ultrastructure, small cell size, dearth of mitochondria, and low contractility, persist in the definitive structure (Bakker, 2010).

Studies of stem cells under reduced gravity conditions both directly and indirectly hint at the developmental pathways that may be involved in sinoatrial nodal development. Indeed, as stated previously, pacemaker cell expansion under simulated microgravity has been observed directly by Hatzistergos et al. (2018) and indirectly in our laboratory (Baio, 2018). Furthermore, the observation by Bressan et al. (2013) that pacemaker cells are specified shortly after gastrulation and prior to the onset of cardiogenesis in a population of Nkx2-5- and Isl1-negative cells is mirrored by a similar reduction in Nkx2.5 and Isl1 expression in CPCs under SMG (Baio, 2018). Moreover, calcium

signaling, which has an integral role in the process of CPC adaptation to spaceflight (Baio, 2018), is activated in the developing cardiac conduction system through Wnt7a (Gessert, 2010), which mobilizes intracellular calcium (Thrasivoulou, 2013). Finally, a recently published study from the laboratory of Ann C. Foley that sought to clarify the mechanism underpinning SAN induction found that MAP3K7, or TGFß-activated kinase 1, overexpression in mouse embryonic stem cells faithfully induced sinoatrial node development (Brown, 2017). Similarly, MAP3K7, is inducible via TGFß signaling, which was found to be induced in CPCs under SMG (Baio, 2018). As an important aside, MAP3K7 activation via TGFß signaling can induce cardiac hypertrophy when overexpressed in myocardium (Zhang, 2000), but apparently promotes sinoatrial nodal development when overexpressed in a sufficiently early progenitor cell. Thus, the application of simulated microgravity-inspired signaling to sinoatrial nodal cell development likely requires an appropriately early progenitor cell.

When considered together, both lines of evidence support the emerging hypothesis that sinoatrial nodal cells represent a de-/un-differentiated state of cardiac development (Bakker, 2010). Thus, findings from the study of CPCs under reduced gravity conditions will serve as the basis for the induction of sinoatrial nodal cells on Earth. In this way, a foundational understanding of the molecular biology and physiology of SAN cells is central to several components of this dissertation.

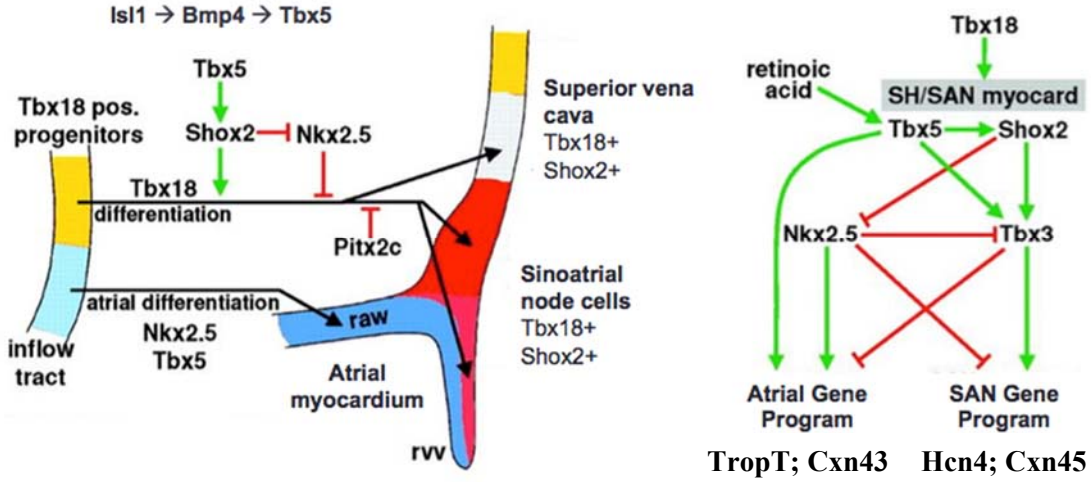

Figure 2. Putative molecular embryology of the SAN (Christoffels, 2010)
Using animal models over previous decades, several key transcriptional factors have been involved in the ultimate expression of the *HCN* family of genes. Yellow indicates mesenchymal precursor tissue while gray indicates sinus horn myocardium and dark blue indicates atrial myocardium. rvv: right venous valve; raw: right atrial wall.

Sinoatrial Node Molecular Development

While most cells that express Nkx2.5 eventually become heart tissue, not all heart cells are Nkx2.5-positive. Indeed, as discussed below, cells that are destined to become the sinoatrial node (Mommersteeg, 2007; Espinoza-Lewis, 2009) have been shown to be absent of Nkx2.5. Although the sinoatrial node is located at the inflow tract that is formed, in part, by the second heart field, it is molecularly unlike the other derivatives of this region of cardiogenic mesoderm. Therefore, whether the second heart field expresses Nkx2.5 is less significant than the observation that its inhibition in the putative tissue of the sinoatrial node allows for the expression of the sinoatrial node gene program. As shown in Figure 2, the Tbx5-mediated induction of Shox2 results in Nkx2.5 inhibition, thereby allowing for Hcn4 and Tbx3 expression and, ultimately, sinoatrial node formation (Munshi, 2012). Furthermore, Tbx3 then inhibits, through Shox mediation, atrial genes, such as Cx40 and Cx43, which encode for connexin proteins, as well as ANF, the atrial natriuretic factor that is involved in the homeostatic control of blood pressure through water and ion secretion regulation (Mommersteeg, 2007; Widmaier, 2008; Boogerd, 2011). Paradoxically, Nkx2.5 expression can function synergistically with Tbx5 to promote the expression of Cx40 and Cx43 in other portions of the cardiac conduction system, such as the atrioventricular node (Munshi, 2012). The location of Shox2 expression participates in determining the mechanism of interaction between Nkx2.5 and Tbx5. In the inflow tract, Tbx5 regulates Shox2 expression, thereby allowing for Nkx2.5 inhibition and Bmp4 expression (Puskaric, 2010), which supports sinoatrial node development. Additionally, Isl1, a gene that is expressed ubiquitously in CPCs, including those of the second heart field (Bu, 2009; Cai, 2003), induces BMP expression,

thereby facilitating Tbx3 expression (Yang, 2006). The ultimately atria-apposed sinoatrial node may also make use of Isl1-expressing tissue of the second heart field-derived right atrium to induce this BMP expression. Despite this relationship between the second heart field and the sinoatrial node gene Tbx3, it has been suggested that the avian sinoatrial node arises from a mass of mesoderm that is not located within, but rather posterior to, the second heart field (Bressan, 2013). This tertiary heart field utilizes Wnt signaling to specify pacemaking cells shortly after gastrulation. Since Wnt is observed to inhibit cardiac development (Bruneau, 2012), it is possible that the necessary inhibition of cardiac features, including gap junctions and atrial natriuretic factor production, for sinoatrial node development (Mommersteeg, 2007) occurs via a Wnt-mediated mechanism in addition to the evidenced role for Tbx3 in inhibiting atrial genes. Alternatively, Wnt suppression, as occurs in the outflow tract due to a gradient of Hopx expression that works with BMP through Smad proteins to suppress Wnt, promotes cardiomyocyte formation (Jain, 2015).

Finally, the right-sided sinoatrial node is prevented from isomerizing (i.e., developing on the left) through the early expression of the Pitx2 homeobox gene (Mommersteeg, 2007). Briefly, Pitx2c positively regulates miR-106b expression, which directly represses sinoatrial node genes, including Shox2 and Tbx3 (Wang, 2014).

For this reason, the sinoatrial node appears to develop in a manner that is molecularly distinct from other cardiac tissue. In fact, it is presently believed to develop from a unique population of heart tissue wherein Wnt expression prevails, thereby participating in the inhibition of the expression of otherwise default cardiac genes (e.g.

Nxk2.5). These unique, non-cardiac features must be considered while identifying precursor cells and developing an *ex vivo* sinoatrial node.

Current Progress in Creating a Biological Pacemaker

Presently, significant progress has been made concerning the isolation and characterization of endogenous CPCs (Bearzi, 2009; Davis, 2009; Fuentes, 2013; Menasché, 2015; Moretti, 2006). Nevertheless, no literature to date has reported the isolation of sinoatrial nodal cells without having first performed an induction technique using an exogenous agent or gene therapy. This may be due to the rarity of the relevant cells. Indeed, the sinoatrial node is only comprised of approximately 10,000 cells that are highly specialized to regulate the contraction of the approximately five billion cardiomyocytes of the human heart (Kapoor, 2013).

In 2004, Rosen *et al.* issued a list of ideal characteristics of a biological pacemaker. Their work highlighted the need for the regenerated pacemaker to respond to autonomic regulation, thereby allowing for the heart to continuously couple cardiac output with a variable metabolic demand. Therefore, it is necessary to ensure that autonomic coupling pathways are preserved in the exogenously produced biological pacemaker.

The *in vivo* transfer of genes has resulted in the successful generation of sinoatrial nodal cells. Tse *et al*. (2006) demonstrated the effectiveness of gene-based therapy for correcting automaticity generation and maintenance by transfecting a mutated form of HCN1, which favors channel opening into guinea pig hearts. Similarly, Potapova et al. (2004) transfected human mesenchymal stem cells with mHCN2 and injected these

transformed cells into the left ventricle of the canine heart, thereby producing left-sided, spontaneous automaticity. Recently, Tbx18 transduction in guinea pig cardiomyocytes resulted in the generation of sinoatrial nodal cells *in vitro* and *in vivo* (Kapoor, 2013).

While these gene-based therapies have reliably produced intrinsic and automated biological pacemaker activity that has limited autonomic sensitivity, their potential contribution to neoplasia has not been documented. For this reason, the use of non-transformed sinoatrial nodal cells may yield clinically-relevant therapies. For example, a recent study that cultured c-kit-positive mouse CPCs in angiotensin II-supplemented growth media reported the promotion of pacemaker-like cell differentiation (Xue, 2015). This study makes use of the presence of angiotensin-II receptors (ATRs) in the developing and adult heart (Hein, 2002). ATRs also are central mediators of cardiac tissue remodeling during heart failure (Tadevosyan, 2012). The angiotensin II receptor is a G-protein coupled receptor that is expressed throughout several types of tissues, including in the conduction system of the heart (Saito, 1987). Furthermore, these receptors are denser in the neonatal heart than in the healthy adult heart (Urata, 1989). The characterization of the angiotensin II receptors in rodent models has shown that its activation produces increases in ERK phosphorylation, nuclear [Ca^{++}] levels, proliferation, and transcription (Tadevosyan, 2012). While the role of angiotensin-II and the ATR in SAN development have not been explored, several researchers have observed effects of angiotensin-II administration on cardiomyocyte electrical properties in rodents. These include decreases in gap junction conductance (De Mello, 1995, 1996) and alterations in the expression of connexin 43 (Severs, 1994). Moreover, intracellular

injection of angiotensin-II decreased the inward calcium current, which suggests the involvement of angiotensin-II in cardiac contractility (De Mello, 1998, 2004).

Meanwhile, another study circumvented the need to induce or transfect cells by excising a porcine sinoatrial node and cultured the homogenized cells on a collagen-based scaffold (Wan, 2009). While this study demonstrated the feasibility of utilizing a scaffold for *ex vivo* cell attachment, no molecular or physiological studies were conducted to confirm the persistence of functional sinoatrial nodes.

These gene therapy-based and chemical cocktail induction strategies have been effective in furthering the understanding of the molecular significance of several families of genes in the sinoatrial nodal cell lineage, they are limited in their clinical suitability. Concerns over neoplasia or ectopic growths of tissue have prevented the wider application of cellular transformation *in vivo*; however, it has been observed that transgenic expression of the reprogramming cytokine TNF-α abolished any neoplastic growths (Behfar, 2007). Similarly, the absence of a relevant preclinical animal model (e.g. sheep or pig) presents a barrier for the eventual use of an exogenously produced sinoatrial node. Furthermore, effective transgenic induction of the growth of sinoatrial nodal cells still only transforms approximately 10%-20% of a progenitor population, while the remaining cells develop into cardiomyocytes. Whether these cells are truly pacemaking (i.e., sinoatrial nodal) cells or simply cardiomyocytes with sinoatrial-nodal like properties requires further characterization and functional testing (Barbuti, 2015).

Recent work by the laboratories of Keller (Protze, 2017) and Foley (Brown, 2017) has produced important advances in the field of sinoatrial nodal development. Both laboratories treated embryonic (the Foley lab) or induced pluripotent (the Keller lab) stem

cells to induce cardiomyocytes that exhibit functional profiles characteristic of spontaneously depolarizing sinoatrial nodal cells. Whereas the former group overexpressed MAP3K7 to achieve this end (Brown, 2017), the latter group titrated Bmp4 treatment at the onset of cardiogenic mesoderm induction to create a sinoatrial nodal-like cell (Protze, 2017). Interestingly, working cardiomyocytes, which exhibit high contractility, exhibit a morphology that differs from that of sinoatrial nodal cells (Bakker, 2010). However, the enriched population of SAN-like cardiomyocytes generated by Brown et al. (2017) and Protze et al. (2017) follows from observations in other labs that such cells can be produced in smaller percentages as a byproduct of inducing ventricular cardiomyocytes from ESCs using a standard protocol of temporally regulated moprhogens (Jha, 2016). Future work is needed to clarify whether such cells are functionally capable of serving as a biological pacemaker over a prolonged period of time, or whether they will revert into a ventricular- or atrial-like cardiomyocyte upon transplantation. Nevertheless, the use of human-derived CPCs as seeds for the development of an *ex vivo* sinoatrial node is possible.

Preamble

Spaceflight and simulated microgravity may promote the efficacious use of stem cells in therapies on Earth. Yet, in the context of cardiovascular progenitor cells (CPCs), the molecular events underpinning adaptation to microgravity remain unknown. The unique extracellular environment fostered by microgravity impacts key aspects of cardiogenesis, modifies early developmental gene expression, and induces expression of the sinoatrial nodal gene program. Therefore, in characterizing the molecular events by

which CPCs adapt to reduced gravity conditions, it is possible to identify key upstream elements that can be used to induce sinoatrial nodal gene expression and promote the development of a biological pacemaker.

Thus, we first identified changes in CPC development and function following spaceflight as well as assessed its implications for CPC applications on Earth. In Chapter 2, we characterize the developmental profile changes of CPCs following culture aboard the International Space Station. We present our findings regarding the key signaling components of the molecular events observed under microgravity and recapitulate portions of these key signaling events on Earth in Chapter 3. These findings are then extended in Chapter 4 to development of sinoatrial nodal cells. The physiology of treated cells, including pacing potential and responsiveness to autonomic regulation via treatment with epinephrine, was then assessed.

In clarifying portions of the molecular events underpinning the response of cardiovascular progenitor cells to reduced gravity conditions, we identify novel avenues for advancing the use of stem cells in regenerative medicine, including biological pacemaker development, on Earth.

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CHAPTER TWO

CULTURE ABOARD THE INTERNATIONAL SPACE STATION ENHANCES THE PROLIFERATIVE POTENTIAL OF CARDIOVASCULAR PROGENITOR CELLS AND INDUCES AN EARLIER DEVELOPMENTAL STATE

This chapter has been previously published in:

Baio JM, Martinez AF, Silva I, Hoen C, Countryman S, Bailey L, Hasaniya N, Pecaut MJ, Kearns-Jonker M. Cardiovascular Progenitor Cells Cultured Aboard the International Space Station Exhibit Altered Developmental and Functional Properties. Submitted.

Abstract

The heart and its cellular components are profoundly altered by missions to space and injury on Earth. Further research, however, is needed to characterize and address the molecular substrates of such changes. For this reason, neonatal and adult human cardiovascular progenitor cells (CPC) were cultured aboard the International Space Station. Upon return to Earth, we measured changes in the expression of microRNAs and of genes related to mechanotransduction, cardiogenesis, cell cycling, DNA repair, and paracrine signaling. We additionally assessed endothelial tube formation, cell cycling, and migratory capacity of CPCs. Changes in microRNA expression were predicted to target extracellular matrix interactions and Hippo signaling in both neonatal and adult CPCs. Genes related to mechanotransduction (*YAP1, RHOA*) were downregulated, while the expression of cytoskeletal genes (*VIM, NES, DES, LMNB2, LMNA*), non-canonical Wnt ligands (*WNT5A, WNT9A*), and Wnt/calcium signaling molecules (*PLCG1, PRKCA*) was significantly elevated in neonatal CPCs. Increased mesendodermal gene expression along with decreased expression of mesodermal derivative markers (*TNNT2, VWF,* and *RUNX2*), reduced readiness to form endothelial tubes, and elevated expression of Bmp and Tbx genes, were observed in neonatal CPCs. Both neonatal and adult CPCs exhibited increased expression of DNA repair genes and paracrine factors, which was supported by enhanced migration. While microgravity affects cytoskeletal organization and migration in neonatal and adult CPCs, only neonatal CPCs experienced increased expression of early developmental markers and an enhanced proliferative potential. Efforts to recapitulate the effects of microgravity on Earth by regulating processes described herein may be a promising avenue for cardiac repair.

Introduction

Changes to the cardiovascular system during spaceflight have prompted molecular biologists to understand the mechanisms of cellular adaptation to microgravity. As a result, researchers have begun to identify therapeutic benefits, such as enhanced stemness, following the application of microgravity (MG) and simulated microgravity (SMG) to a variety of stem cell types. Therefore, increased understanding of the response of cardiovascular progenitor cells (CPCs) to microgravity may not only benefit human health in space, but also provide insights into novel cardiovascular stem cell therapies that can be applied on Earth.

Recent research in our own laboratory has shown that neonatal human CPCs cultured under SMG exhibit increased expression of markers of early cardiovascular development and pluripotency (i.e., *MESP1*, *T* (Brachyury), and *POU5F1* (OCT4)) as well as broad changes to microRNA expression (Fuentes, 2015). The changes in microRNA expression experienced by CPCs under SMG mirrored the expression of similar microRNAs in human embryonic stem cells (ESC) under normal conditions of early cardiovascular development, thereby suggesting that SMG induces an epigenetic environment that favors an earlier development status. Such a state appears increasingly beneficial for cardiovascular repair. For example, the efficacy of multipotent stem cells expressing $Isl1^{+}$ and SSEA-1^{+} in ameliorating the symptoms of severe heart failure was recently demonstrated in one patient (Menasché. 2015). Furthermore, KEGG analysis indicated that the microRNAs down-regulated in CPCs by SMG target several signaling pathways, such as MAPK and Wnt, which are relevant to well-characterized repair mechanisms or cardiac stem cell proliferation mechanisms in the heart. For example,

non-canonical Wnt ligands (i.e., Wnt2b, Wnt5a, and Wnt9a) have been shown to be associated with the injury response in neonatal rodent heart tissue (Mizutani, 2016). Therefore, our observations suggest that SMG may foster a developmental state and signaling events that could enhance the use of CPCs for cardiovascular repair. This is supported by one recent study by Jha et al. (2016), which demonstrated that human induced pluripotent stem cells more readily differentiate into cardiomyocytes following three-dimensional (i.e., embryoid) culture coupled with SMG. In the context of our findings, these experiments may highlight a potential phenomenon in which a low gravity culture promotes an enhanced state of stemness that results in increased differentiation potential when the cells are returned to normal gravity conditions.

It is currently believed that small RhoGTPases act as transducers of the mechanical alterations that are observed under conditions of microgravity (Meyers, 2005; Louis, 2015). As actors in the planar cell polarity Wnt pathway, they have central roles in cytoskeletal remodeling. Along with the Hippo pathway and calcium signaling, small RhoGTPases represent one potential molecular substrate of gravity sensing. Notably, in the context of cardiogenesis, these processes are central to maintaining multipotency or directing differentiation (Pucéat, 2005). Despite the early reports of the effects of microgravity on stem cells, much is unknown about the mechanisms by which CPCs respond to a reduced gravity environment or how these cell types develop enhanced stemness. Hence, we are not yet able to leverage the therapeutic potential of altered gravity conditions here on Earth. While current clinical trials to stimulate repair in damaged heart tissue are promising (Bolli, 2011; Makkar, 2012; Gerbin, 2015), they are stymied by a failure of cells to engraft into the host tissue and by the use of progenitor

types that are restricted in potency (Hong, 2014). Therefore, identifying the molecular events that promote enhanced stemness and regenerative potential in CPCs under reduced gravity conditions will benefit stem cell-based cardiac repair.

The successful use of autologous CPC sources to regenerate heart tissue in a predominately adult population requires additional understanding of the differences between adult and neonatal CPCs. Our laboratory has previously documented differences in age-dependent expression of microRNAs related to MAPK signaling, cytoskeleton regulation, adherens junction expression, and focal adhesion maintenance (Fuentes, 2013). The relationship between these processes and the age-dependent response of CPCs to reduced gravity remain unknown, but they imply deficits in the intracellular transduction of environmental cues in the functional shortcomings of adult CPCs.

Therefore, in this study, clonal lines of neonatal and adult human CPCs were cultured aboard the International Space Station (ISS). We sought to identify changes in these CPCs in response to the reduced gravity conditions that impact signaling, development, and stemness. We then characterized the divergent response of neonatal and adult CPCs to spaceflight. We present components of the adaptive cellular response to a reduced gravity environment and their implications for enhancing the regenerative potential of neonatal and adult CPCs.

Materials and Methods

Ethics Statement/Cell Isolation and Expansion

CPCs were isolated from cardiac tissue of neonates (1 day – 1 month) or adults (57 – 72 years), as previously described (Fuentes, 2013). The Institutional Review Board

of Loma Linda University approved the protocol for use of tissue that was discarded during cardiovascular surgery, without identifiable private information, for this study with a waiver of informed consent. Briefly, atrial tissue was cut into small clumps (approximately 1.0 mm^3) then enzymatically digested using collagenase (Roche, Indianapolis, IN) at a working concentration of 1.0 mg/mL. The resulting solution was then passed through a 40-µm cell strainer. Cells were cloned in a 96-well plate by limiting dilution to a final concentration of 0.8 cells per well to create populations for expansion. Cells were then screened for the co-expression of Isl1 and c-Kit. Clonal CPC cultures were selected for other markers of early cardiac development (KDR and PDGFRA) and then maintained using growth media comprised of 10% fetal bovine serum (Thermo Scientific, Waltham, MA), 100 µg/mL penicillin-streptomycin (Life Technologies, Carlsbad, CA), 1.0% minimum essential medium non-essential amino acids solution (Life Technologies, Carlsbad, CA), and 22% endothelial cell growth media (Lonza, Basel, Switzerland) in Medium 199 (Life Technologies, Carlsbad, CA). Mycoplasma contamination was tested using the MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland) following the manufacturer's protocol.

Developmental Marker Detection Using Flow Cytometry

Progenitor cell populations were fluorescently labeled with antibodies, as recommended by their respective manufacturers, and then analyzed using a MACSQuant® analyzer (Miltenyi Biotec, Auburn, CA). Briefly, for developmental marker detection, CPCs were incubated with Viobility 405/520 before being washed in 1X PBS (Life Technologies, Grand Island, NY) containing 0.5% BSA (Research

Products International Corp, Mt. Prospect, IL) and 2mM EDTA (Sigma Aldrich, St. Louis, MO). Then, CPCs were stained for cardiac-related stem cell surface markers (PDGFRα, CXCR4, c-Kit, and SSEA1). CPCs were then fixed in 4% PFA (Sigma Aldrich, St. Louis, MO), permeabilized in 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO), blocked in 0.6 M glycine (Sigma Aldrich, St. Louis, MO) solution containing 10% BSA (Research Products International Corp, Mt. Prospect, IL), and stained for intracellular, cardiac-related stem cell surface markers (Isl1 and Mesp1). For directed differentiation assays, cells were stained for TropT after being fixed in 4% PFA (Sigma Aldrich, St. Louis, MO), permeabilized in 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO) or directly for vWF or SMA. Stained CPCs were then analyzed using a MACSQuant® analyzer (Miltenyi Biotec, Auburn, CA). Quantification of data was performed using FlowJo software version 10 (Ashland, OR). Compensation was performed using UltraComp eBeads (Life Technologies, Grand Island, NY) following the manufacturer's protocol. Briefly, one drop of compensation beads were incubated with each antibody individually for 30 minutes at 4ºC and then detected using the same MACSQuant® analyzer just prior to experiments requiring compensation. FlowJo was then used to generate a compensation matrix, which was applied to all experimental data. Information concerning antibodies, isotype controls, and reagents used in antibody labeling is provided in Table 3.

Directed Differentiation of Cardiovascular Progenitor Cells

To validate the ability of neonatal and adult CPCs to differentiate into cells of the cardiovascular lineage, directed differentiation assays were performed, as described in Le

et al. (2018) and Smits et al. (2009). In brief, to generate cardiomyocytes, CPCs were cultured in growth media (see above) until 85% confluent. CPC growth media was then replaced with differentiation media (47% IMDM, 47% Ham's F12 with GlutaMAX, 2.0% penicillin-streptomycin, 2.0% horse serum, 1.0% minimum essential medium non-essential amino acids solution, and 1.0% insulin-transferring selenium (all reagents from Life Technologies, Carlsbad, CA) containing 5 μM 5-Azacytidine (Sigma, St. Louis, MO), which was refreshed daily for three days. Then, 1 ng/mL TGF-β (R&D Systems, Minneapolis, MN) and 0.1 mM ascorbic acid (Acros Organics, Geel, Belgium) in fresh differentiation media was added routinely until day 14, at which time cells were stained for TropT or placed in TRIzol® reagent (Life Technologies, Carlsbad, CA) for gene expression analysis. To generate endothelium, CPCs were cultured in growth media (see above) until 85% confluent, at which time the growth media was replaced with endothelial cell growth media (Lonza, Basel, Switzerland) containing 25 ng/mL Bmp4 (R&D Systems, Minneapolis, MN) and 50 ng/mL Vegf (R&D Systems, Minneapolis, MN) for two days. The media was then replaced with endothelial cell growth media (Lonza, Basel, Switzerland) containing 50 ng/mL Vegf and cultured for four days. Then, cells were regularly fed endothelial cell growth media (Lonza, Basel, Switzerland) until day 14, at which time cells were stained for vWF, placed in TRIzol® reagent (Life Technologies, Carlsbad, CA) for gene expression analysis, or seeded onto Matrigel for tube formation assay analysis, as described below. Finally, to generate smooth muscle cells, CPCs were cultured in growth media (see above) until 85% confluent, at which time the growth media was replaced with DMEM (Life Technologies, Carlsbad, CA) containing 4.5 g/L D-glucose, 2% fetal bovine serum (Thermo Scientific, Waltham, MA),

and 50 ng/mL PDGF-BB (R&D Systems, Minneapolis, MN). This media was regularly replaced until day 14, at which time cells were stained for smooth muscle actin or placed in TRIzol® reagent (Life Technologies, Carlsbad, CA) for gene expression analysis. Antibodies used for flow cytometry analysis are included in Table 4.

Biocell Seeding and Spaceflight

Neonatal and adult CPCs were seeded into Biocells (BioServe Space Technologies, Boulder, CO) at two seeding densities (7,500 cells or 5,000 cells) and loaded into self-contained environments containing 5% CO_2 and 95% air. They were then flown aboard SpaceX CRS-11 to the US National Lab on the ISS where they were placed in an incubator containing 5% CO_2 and 95% air. Fresh media was provided every four to five days while aboard the ISS (Figure 5). At 12 days, the Biocells that were seeded with 7,500 cells were flooded with RNA Protect (Qiagen, Valencia, CA) and stored at -80ºC. At 30 days, the Biocells seeded with 5,000 cells were fed and returned to Earth. Clone- and passage-matched ground controls were fed and treated in parallel with the feeding schedule and activities performed by astronauts aboard the ISS.

Post-Flight Sample Processing

Upon landing and retrieval of the payload, live cells were trypsinized, counted, and used to assess cell cycle, migration, and endothelial tube formation, as well as to prepare protein lysates. Biocells containing cells fixed and frozen in RNA Protect were thawed at room temperature. The RNA Protect was removed and centrifuged at 10,000g at 4ºC for 10 minutes. Biocells were disassembled and the culture membranes were then

rinsed with TRIzol® reagent (Life Technologies, Carlsbad, CA). RNA was purified from the RNA protect samples using the RNeasy Mini Kit (Qiagen, Valencia, CA), per the manufacturer's instructions, while total RNA was purified from TRIzol® reagent using isopropanol- and ethanol-based precipitation. cDNA was generated and RT-PCR was performed as described below.

RT^2 MicroRNA Profiler Array and miRNA RT-PCR

MicroRNA array profiling was performed as previously described (Fuentes, 2015). Briefly, the miScript II RT Kit (Qiagen, Valencia, CA) was used to convert 500 ng of total RNA into cDNA, which was diluted, added to miScript SYBR Green PCR master mix and 10X universal primer mix (Qiagen, Valencia, CA), and run on human development and differentiation miScript plates (MIHS-103ZA; Qiagen, Valencia, CA). Fold changes were determined for each clone using the Qiagen Data Analysis Center (http://www.qiagen.com/us/shop/ genes-and-pathways/data-analysis-center-overview-page/) using all available housekeeping genes. Since this analysis center performs only a two-tailed Student's t-test to calculate P-values, all fold changes for individual clones were exported to Prism and analyzed, as described below. Individual primers for SNORD96a, SNORD72, hsa-miR-100-5p, and hsa-miR-99a-5p (SABiosciences, Valencia CA) were also used (Table 5). The average C_t value of SNORD96a and SNORD72 was used as a housekeeping control. The PCR conditions for the microRNA arrays and individual microRNA assays were: 95°C for 15 minutes and 40 cycles of 94°C for 15 seconds, 55°C for 60 seconds, and 70°C for 30

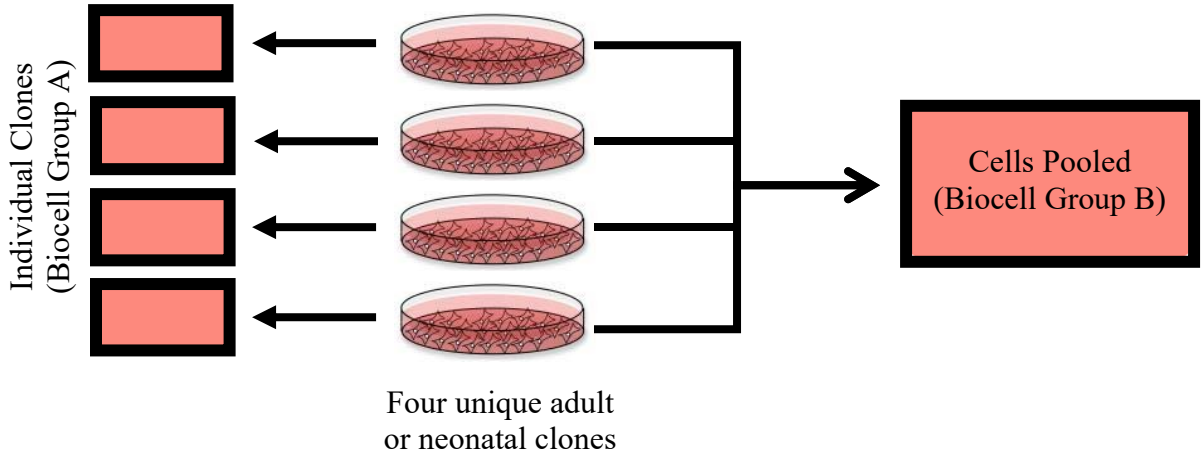
Table 3. Antibodies used to identify CPCs for experiments aboard the ISS

Antibody	Manufacturer	Isotype	Conc.	Species	Clone	Cat. No.	Lot No.
Mesp1-DyLight 405	Novus Biologicals	IgG	0.8mg/mL	Rabbit	2030B	MAB9219V	CKNQ01-1-050317-V
Isotype-DyLight405	Novus Biologicals	IgG	1.05mg/mL	Rabbit	NBP2-36463V	NBP2-36463V	31908-050317-V
Viobility 405/520	Miltenyi Biotec	n/a	n/a	n/a	n/a	130-109-814	5170315796
Islet1	Abcam	IgG1	0.83mg/mL	Mouse	1H9	ab86472	GR273015-3
Fluorescein Labeling Kit	Novus Biologicals	n/a		n/a	10934	707-0030	1BS3384
Isotype-FITC	BioLegend	IgG1 kappa	0.5mg/mL	Mouse	MOPC-21	400107	B199152
PDGFRα-PE	BioLegend	IgG1 kappa	100ug/mL	Mouse	16A1	323505	B192368
Isotype-PE	BioLegend	IgG1 kappa	200ug/mL	Mouse	MOPC-21	400113	B214532
CXCR4-PE/Vio770	Miltenyi Biotec	IgG1	82.5ug/mL	n/a	REA649	130-109-887	5170503056
REA CtRl-PE/Vio770	Miltenyi Biotec	IgG1	20ug/mL	n/a	REA293	130-104-616	5170201559
cKit-DyLight650	Novus Biologicals	IgG2B kappa	500ug/mL	Mouse	2B8	NB100-77477C	B147020-A
Isotype-AlexaFluor 647	R&D Systems	IgG2B	10ug/mL	Rat	141945	IC013R	AEIU0114121
SSEA1-APC/Vio770	Miltenyi Biotec	IgG1	8.25ug/mL	n/a	REA321	130-104-992	5161207356
REA Ctrl-APC/Vio770	Miltenyi Biotec	IgG1	30ug/mL	n/a	REA293	130-104-618	5170201560

Table 4. Antibodies used in directed differentiation assays

Antibody	Manufacturer	Isotype	Conc.	Species	Clone	Cat. No.	Lot No.
TropT-PE	Miltenyi Biotec	IgG1	10 µg/mL	n/a	REA400	130-106-746	5160426814
Isotype-PE	BD Pharmigen	IgG1 kappa	0.5 mg/mL	Mouse	MOPC-21	556650	19587
vWF-AlexaFluor 657	Novus Biologicals	IgG1 kappa	0.75 mg/mL	Mouse	3E2D10	NBP2-34535AF647	7450-1PABX160519120816-AF647
Isotype-AlexaFluor 647	R&D Systems	IgG2B	10 µg/mL	Rat	141945	IC013R	AEIU0114121
αSMA-PerCP	Novus Biologicals	IgG2a kappa	0.4 mg/mL	Mouse	1A4/asm1	NBP2-34522PCP	59-1PABX170619-090617-PCP
Isotype-PerCP	Miltenyi Biotec	IgG1	55 µg/mL	Mouse	IS5-21F5	130-094-968	5110906188

Biocell Seeding



Experiment Timeline

Day 0	Day 3	Day 8	Day 12	Day 16	Day 20
Launch (37ºC & 5% CO_2)	Arrives to ISS Media Change (Groups A & B)	Media Change (Groups A & B)	Fixation (Biocell Group A) Media Change (Biocell Group B)	Media Change (Biocell Group B)	Media Change (Biocell Group B)

Day 24	Day 27	Day 30	Day 31 – Return to Lab
Media Change (Biocell Group B)	Media Change (Biocell Group B)	Return to Earth (37ºC & 5% CO_2)	<u>Group A</u> RNA Purification <u>Group B</u> Endothelial Tube Assay Cell Cycle Analysis Migration Assay Protein Isolation RNA Purification

Figure 3. ISS Experiment Schematic
An outline of the experimental design for culture aboard the ISS, including how cell culture systems were prepared prior to launch and a timeline of experiments once aboard the ISS.

seconds. Differences in fold changes between ground- and ISS- cultured CPCs were then analyzed as described in the statistics sub-section.

MicroRNA Targeting Prediction

KEGG analysis was performed using DIANA mirPath version 3 and TarBase v7.0 for microRNAs that were found to be significantly altered in neonatal and adult CPCs. Results were merged by pathway union after controlling for error rate due to multiple comparisons via selecting the "correct for FDR" feature. After removing categories related to cancer, neurological diseases, and viruses/pathogenesis, the KEGG categories whose p-values were less than 0.05 were reported.

RT^2 Profiler Array

We used custom array plates (CLAH22469A; Qiagen, Valencia, CA) per the manufacturer's instructions to measure gene expression changes in ISS-cultured adult and neonatal CPCs that were relevant to Wnt, ERK, BMP/Smad, and Notch signaling; cytoskeletal maintenance; calcium handling; apoptosis and cell cycle; cardiac development and stemness; and regeneration. Briefly, 2 μg of RNA was reverse transcribed into cDNA, as described above, and then thoroughly mixed with 2X RT^2 SYBR Green Mastermix and RNase- and DNase-free water before being loaded into the profiler array plate. Samples were amplified in the iCycler iQ™5 PCR Thermal Cycler (Bio-Rad, Hercules, CA) using a protocol of 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for one minute. Fold changes were determined for each clone individually using the Qiagen Data Analysis Center (http://www.qiagen.com/

Table 5. **Primer pair sequences used in RT-PCR experiments**
The catalog number, manufacturer, and name of microRNA primers that were not originally included in microarray plates as well as gene names and sequences (reported 5' to 3') included in experiments.

Gene	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
ACTA2	AGCTTTCAGCTTCCCTGAACA	TACAGAGCCCAGAGCCATTG
ACTIN	TTTGAATGATGAGCCTTCGTCCCC	GTCTCAAGTCAGTGTACAGGTAAGC
ATM	GGGCGAGCCGCAAACGCTAA	TTCGGCCCGTCGGAGCAAAC
E2F1	GACCATCAGTACCTGGCCGAGAG	GACGACACCGTCAGCCGAGTG
HGF	CACGAACACAGCTTTTTGCC	TGATCCCAGCGCTGACAAAT
HMOX1	CTCTCGAGCGTCCTCA	ACTATCAGACAATGTTGT
HSP70	TGACCAAGATGAAGGAGATCG	GTCAAAGATGAGCACGTTGC
IGF1	CAGAGCAGATAGAGCCTGCG	CAGGTAACTCGTGCAGAGCA
MESP1	TAGGCCTCAGCGAGGAGAGT	TCCCTTGTCACTTGGGCTCC
MLC2V	GGTGCTGAAGGCTGATTACGTT	TATTGGAACATGGCCTCTGGAT
MYH11	CAAATACGCGGATGAGAGGGA	CTCATGGACGTTCTTGCCCA
NKX2-5	CGCCGCTCCAGTTCATAG	GGTGGAGCTGGAGAAGACAGA
PECAM1	AACGGAAGGCTCCCTTGATG	TAAGAACCGGCAGCTTAGCC
POU5F1	AACCTGGAGTTTGTGCCAGGGTTT	TGAACTTCACCTTCCCTCCAACCA
RAD23A	GTATCGGAGCAGCCGGCCAC	TCCCCAGGGGGCTCGTTCAG
RAD50	CTACGGCTTTGCGTCCCCGG	ACACCAGCTTTCCCCGC
SDF1A	CTACAGATGCCCATGCCGAT	GTGGGTCTAGCGGAAAGTCC
SOX2	AACCAGCGCATGGACAGTTA	GACTTGACCACCGAACCCAT
TERT	AGAGTGTCTGGAGCAAGTTGC	CGTAGTCCATGTTCACAATCG
TNNT2	GTGGGAAGAGGCAGACTGAG	ATAGATGCTCTGCCACAGC
TP53INP1	CACAACAACAAAAGGACTTGGACT	TTGAGCTTCCACTCTGGGAC
TXNIP	TCAGTATTGCAGGGCTTGGC	GTCTCTTGAGTTGGCTGGCT
VEGFA	CAGCGAAAGCGACAGGGGCA	GCTGGAGCACTGTCTGCGCA
VWF	ACACCTGCATTTGCCGAAAC	ATGCGGAGGTCACCTTTCAG
YAP1	TCCCAGATGAACGTCACAGC	TCATGGCAAAACGAGGGTCA

Symbol	MicroRNA	Catalog Number	Manufacturer
hsa-miR-99a-5p	Hs_miR-99a_2	MS00032158	Qiagen
hsa-miR-100-5p	Hs_miR-100	Ms00031234	Qiagen
SNORD72	Hs_SNORD72_11	MS00033719	Qiagen
SNORD96A	Hs_SNORD96A_11	MS00033733	Qiagen

us/shop/genes-and-pathways/data-analysis-center-overview-page/) using *GAPDH* and *ACTB* (β-actin) as housekeeping genes. Since this analysis center performs only a two-tailed Student's t-test to calculate P-values, all fold changes for individual clones were exported to Prism and analyzed, as described below.

Quantitative RT-PCR

Changes in the expression of select genes were analyzed using RT-PCR. RNA was isolated from CPCs as described above. cDNA was prepared using 2 μg of RNA with Superscript III (Life Technologies, Carlsbad, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Go-Taq® qPCR Mastermix (Promega, Madison, WI) and the iCycler iQ™5 PCR Thermal Cycler (Bio-Rad, Hercules, CA) following a protocol of 94°C for 10 minutes and 45 cycles of 94°C for 15 seconds, 52–68°C (depending on the primer) for 60 seconds, and 72°C for 30 seconds. RT-PCR products were visualized using 1–2% agarose gel electrophoresis and low mass DNA ladder (Invitrogen, Carlsbad, CA). Primers were designed using the National Center for Biotechnology Information Primer-BLAST program and obtained from Integrated DNA Technologies (Coralville, IA). Primers used in experiments are listed in Table 5.

Endothelial Tube Formation Assay

Matrigel (Trevigen, Gaithersburg, MD) was added to a 96-well plate (50μL/well) and hardened in a humidified, 5% CO_2 incubator at 37ºC for 1 hour. Cells (20,000/well) were then incubated for seven hours in EGM-2 media (Lonza Allendale, NJ) before being

stained with Calcein AM (Fisher Scientific, Pittsburg, PA) for 30 minutes. Their ability to form capillary-like networks was measured using an EVOS imaging system (ThermoFisher Scientific, Waltham, MA) and quantified with ImageJ (v1.47f, NIH, Bethesda, MD).

Cell Cycle Analysis

Aliquots of 250,000 cells were fixed with 70% ethanol overnight, incubated for 60 minutes with RNAse A (Fisher Scientific, Pittsburg, PA), and stained with propidium iodide, prior to running samples on a MACSquant analyzer (Miltenyi Biotec, Auburn, CA) and analyzing cell cycle progression using the Dean-Jett-Fox model in the cell cycle analysis tool of FlowJo software (Ashland, OR).

Migration Assay

CPCs were trypsinized, counted, and resuspended in starving medium that contained IMDM with GlutaMAX (ThermoFisher Scientific, Waltham, MA), 1% Insulin-Transferrin-Selenium (Life Technologies, Carlsbad, CA), and 0.5% FBS (Thermo Scientific, Waltham, MA). 50,000 CPCs were plated in the top chamber of a 96-well transwell migration assay (Corning, Union City, CA) with 8 micron pores. Standard CPC growth medium supplemented with 100ng/mL of SDF-1α (Life Technologies, Grand Island, NY) was used in the bottom chamber as a chemoattractant. After 6 hours, migrated cells in the bottom chamber were stained using Calcein AM (Fisher Scientific Pittsburg, PA) and quantified using a FLX800 fluorescent plate reader (Bio-Tek, Winooski, VT).

Statistical Analysis

The Shapiro-Wilk test for normality was used to test the normality of data distribution. We then performed a two-tailed, paired t-test to compare the mean of all normally distributed data. Non-normally distributed data were compared using a Wilcoxon matched-pairs signed rank text. For cell cycle, migration, and endothelial tube formation assay, samples in each group were pooled and either a two-tailed, unpaired t-test or Mann-Whitney U test was used to compare the mean of normally or non-normally distributed data, respectively. All data are reported as the mean ± the standard error of the mean. Prism 7 version 7.02 (GraphPad, La Jolla, CA) was used for all statistical analyses. P values < 0.05 were assumed to indicate statistical significance.

Results

CPCs Exhibit Markers of Early Mesodermal Development

CPCs were clonally isolated, expanded, screened for the co-expression of Isl1 and c-Kit, assessed for viability, and ultimately selected for experiments based upon the expression of early developmental markers (Mesp1, PDGFRα, and SSEA1) along with the chemokine receptor CXCR4. Representative adult and neonatal clones are shown in Figure 4. Clonal lines of CPCs obtained from neonatal and adult patients were then flown aboard the ISS for 12 days and fixed in RNAprotect during orbit (Figure 3).

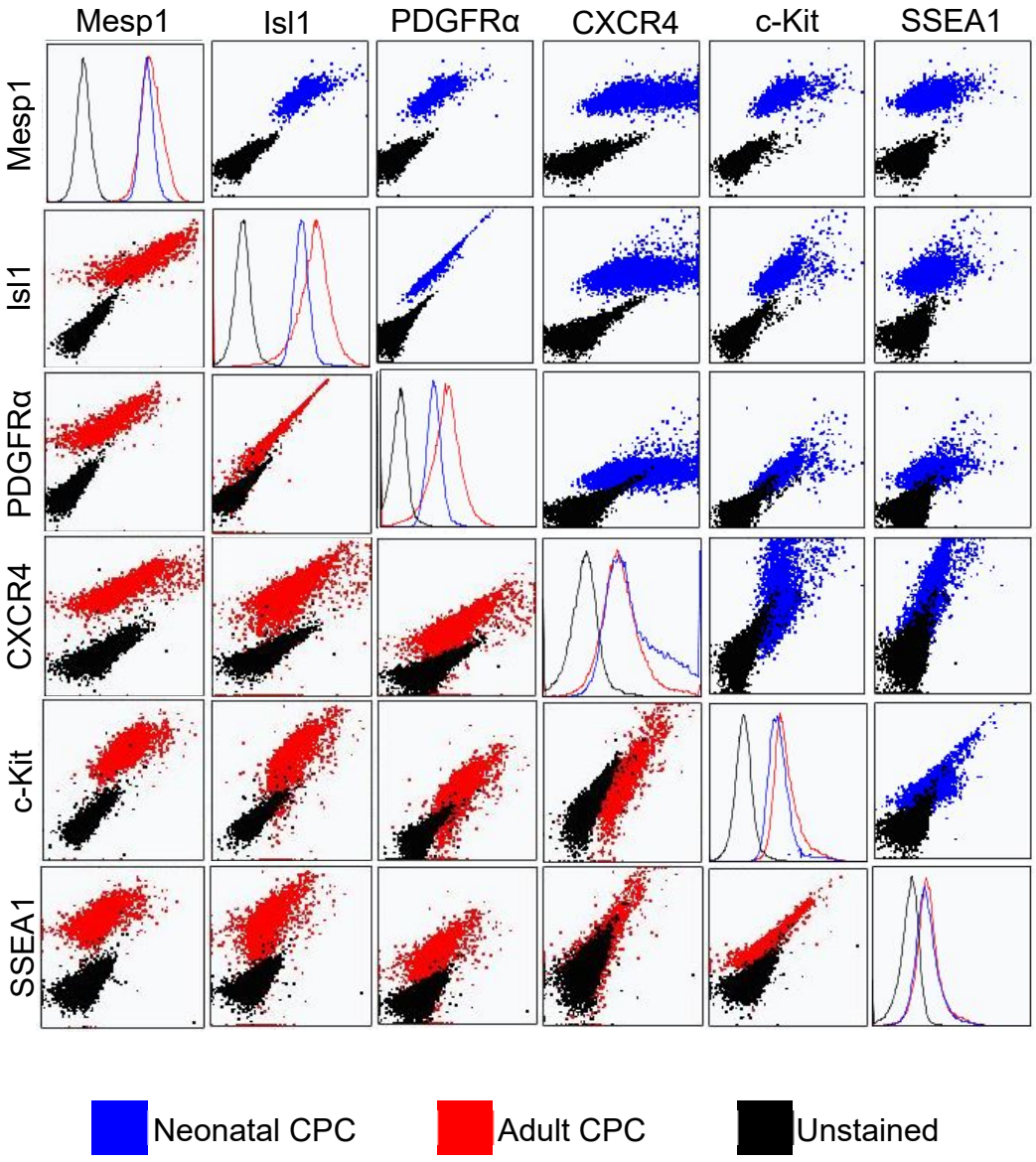


Figure 4. CPCs express markers of early mesodermal development
Neonatal (blue) and adult (red) CPC clones were screened for the co-expression of Isl1 and c-Kit, assessed for viability, and then ultimately selected for experiments based upon the co-expression of early developmental markers (Mesp1, PDGFRα, KDR, and SSEA1) along with the chemokine receptor CXCR4.

CPCs Differentiate Into All Three Major Cardiovascular Lineages

To validate the identity of neonatal and adult CPCs, cells expressing markers of early mesoderm were treated with directed differentiation protocols for two weeks before being assessed for relvant gene and protein expression using RT-PCR and flow cytometry, respectively (Figure 5A). CPCs were treated with 5-azacytidine and Tgfβ to induce cardiomyocytes, which subsequently expressed elevated levels of cardiomyocyte markers *TNNT2, MLC2V,* and the protein TropT (Figure 5B-C). Separately, CPCs were treated with Vegfa, Bmp4, and endothelial growth media to induce endothelial cells, which subsequently expressed elevated levels of endothelial markers *VWF, PECAM1*, and the protein vWF (Figure 5D-E). Endothelial tubes were then formed from endothelial cells (Figure 5F). Finally, cells were treated with Pdgf-BB in DMEM containing 4.5 g/L glucose to induce smooth muscle cells, which subsequently expressed elevated levels of smooth muscle cell markers *ACTA2, MYH11,* and the protein αSMA (Figure 5G-H).

Microgravity Mediates Changes to Expression of microRNAs That Target Membrane Synthesis and Extracellular Interactions

In an effort to understand the relevant signaling events of a reduced gravity environment, we performed microarray analysis to measure broad alterations to transcriptional control. Of the 88 microRNAs analyzed, 14 exhibited significant alterations in levels of expression among neonatal and adult CPCs (n=3 unique clones per age per group for all miRs, except miR-99a and miR100, which were measured in biological and technical triplicates, Figure 6). KEGG analysis by age group (Table 6)

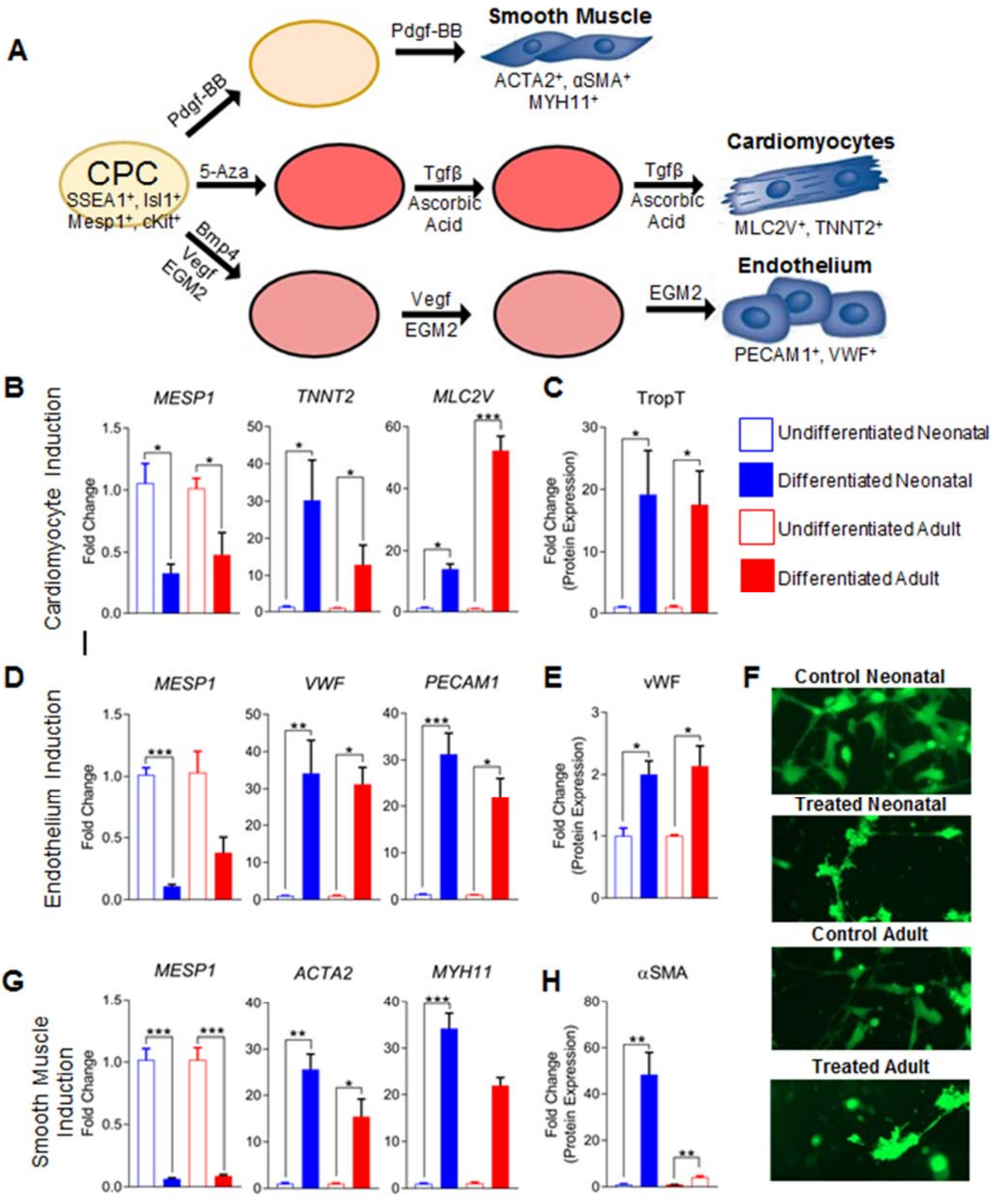

A
Smooth Muscle
Pdgf-BB
ACTA2+, αSMA+
MYH11+
Pdgf-BB
CPC
SSEA1+, Isl1+
Mesp1+, cKit+
5-Aza
Tgfβ
Ascorbic Acid
Tgfβ
Ascorbic Acid
Cardiomyocytes
MLC2V+, TNNT2+
Bmp4
Vegf
EGM2
Vegf
EGM2
EGM2
Endothelium
PECAM1+, VWF+
B
Cardiomyocyte Induction
MESP1
TNNT2
MLC2V
Fold Change
C
TropT
Fold Change (Protein Expression)
Undifferentiated Neonatal
Differentiated Neonatal
Undifferentiated Adult
Differentiated Adult
D
Endothelium Induction
MESP1
VWF
PECAM1
Fold Change
E
vWF
Fold Change (Protein Expression)
F
Control Neonatal
Treated Neonatal
Control Adult
Treated Adult
G
Smooth Muscle Induction
MESP1
ACTA2
MYH11
Fold Change
H
αSMA
Fold Change (Protein Expression)

Figure 5. Neonatal and adult CPCs can differentiate into all major cardiovascular lineages

To validate the identity of CPCs used in experiments, directed differentiation protocols were used for cardiomyocyte, endothelial, and smooth muscle pathway induction (A). Cardiomyocyte (B-C), endothelial cell (D-E), endothelial tube (F), and smooth muscle cell (G-H) induction was screened after 14 days by RT-PCR and flow cytometry. Gene expression patterns for the mesodermal marker *MESP1* and differentiation markers were measured in each unique differentiation experiment. n=3–9 replicates. Data: mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

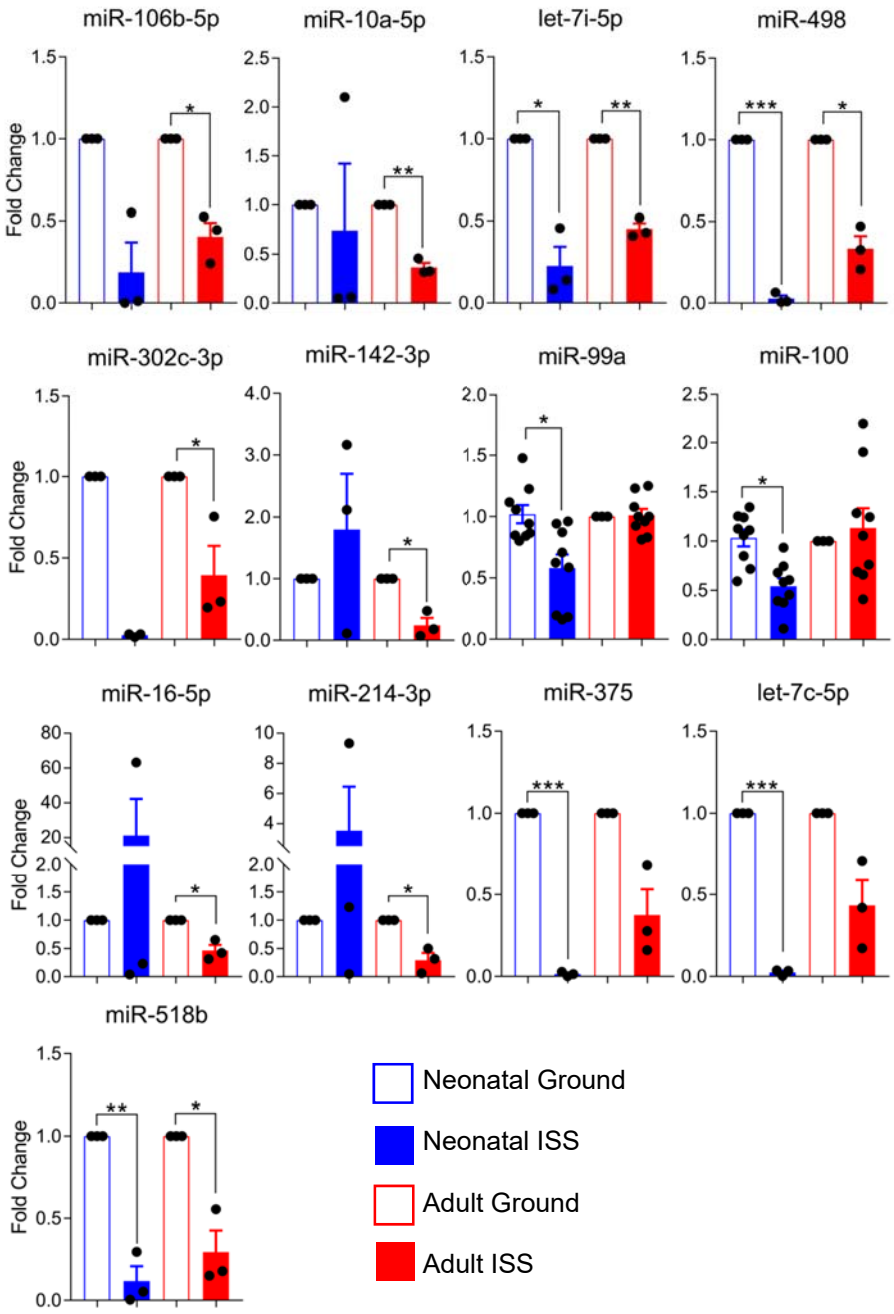


Figure 6. Microgravity alters microRNA expression in neonatal and adult early CPCs Neonatal and adult CPCs both exhibited significant alterations in microRNA expression following 12 days of culture aboard the ISS. KEGG analysis (Table 6) indicated likely targeting of ECM interactions, membrane metabolism, and Hippo signaling by these microRNAs. n=3–9. Data: mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

Table 6. KEGG analysis of targets of significantly altered microRNAs
Following spaceflight, neonatal and adult CPCs were screened for changes to microRNA expression, which were then assessed using KEGG pathway analysis.

Neonatal CPC KEGG Analysis Results

KEGG pathway	P-Value	Genes	miRNAs
Fatty acid biosynthesis	0	2	1
ECM-receptor interaction	0	14	2
Hippo signaling pathway	1.94E-13	43	3
Cell cycle	1.56E-05	43	3
Lysine degradation	2.80E-05	16	2
Glycosphingolipid biosynthesis - lacto and neolacto series	0.000139519	2	2
p53 signaling pathway	0.002223088	25	3
Protein processing in endoplasmic reticulum	0.02048608	51	2
Endocytosis	0.02304169	52	2
Thyroid hormone signaling pathway	0.04192693	34	2

Adult CPC KEGG Analysis Results

KEGG pathway	P-Value	Genes	miRNAs
Fatty acid biosynthesis	0	4	2
ECM-receptor interaction	0	19	4
Hippo signaling pathway	0	87	8
Lysine degradation	1.11E-16	28	6
Adherens junction	1.26E-10	51	5
Cell cycle	2.97E-09	72	3
TGF-beta signaling pathway	3.00E-08	49	5
Protein processing in endoplasmic reticulum	4.16E-08	99	4
Fatty acid metabolism	3.36E-06	16	2
p53 signaling pathway	2.40E-05	41	4
Endocytosis	0.000157412	76	3
FoxO signaling pathway	0.000502794	51	3
RNA transport	0.0013397	73	2
Thyroid hormone signaling pathway	0.003808595	48	3
Estrogen signaling pathway	0.003832405	29	3
Ubiquitin mediated proteolysis	0.00920295	70	3
Signaling pathways regulating pluripotency of stem cells	0.009475487	56	3
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	0.01175345	9	2

indicated that fatty acid biosynthesis, extracellular matrix (ECM)-receptor interactions, and Hippo signaling were the most significantly affected targets of these microRNAs.

Cytoskeletal Maintenance Is Altered in Neonatal, But Not Adult, CPCs Under Microgravity

Since the pathways targeted by the significantly dysregulated microRNAs affect cytoskeletal remodeling and mechanotransduction pathways (Figure 7A), we sought to measure changes in the expression of *YAP1*, a gene that encodes a critical Hippo signaling molecule; small RhoGTPases; and cytoskeletal molecules. *YAP1* was significantly down-regulated in neonatal CPCs (fold change: 0.243 ± 0.833, n=3 unique clones each measured in triplicate per group, P=0.004; Figure 7B) and modestly upregulated in adult CPCs (fold change: 2.629 ± 0.186, n=2 unique clones each measured in triplicate per group, P=0.006; Figure 8A). Among small RhoGTPases, only RhoA was significantly downregulated in neonatal CPCs (fold change: 0.152 ± 0.087, n=3 unique clones per group, P=0.010; Figure 7C), while the cytoskeletal genes *VIM, NES, DES, LMNB2,* and *LMNA* were all significantly upregulated (fold changes: 5.180 ± 1.227, P=0.031; 21.460 ± 5.729; P=0.023; 6.005 ± 1.447, P=0.026; 5.096 ± 1.163, P=0.024; 10.420 ± 2.930, P=0.033; respectively; n=3 unique clones per group) following spaceflight (Figure 7D). Meanwhile, in adult CPCs, only *NES* expression decreased significantly (fold-change: 0.279 ± 0.137, n=3 unique clones per group, P=0.006) (Figure 8B & C).

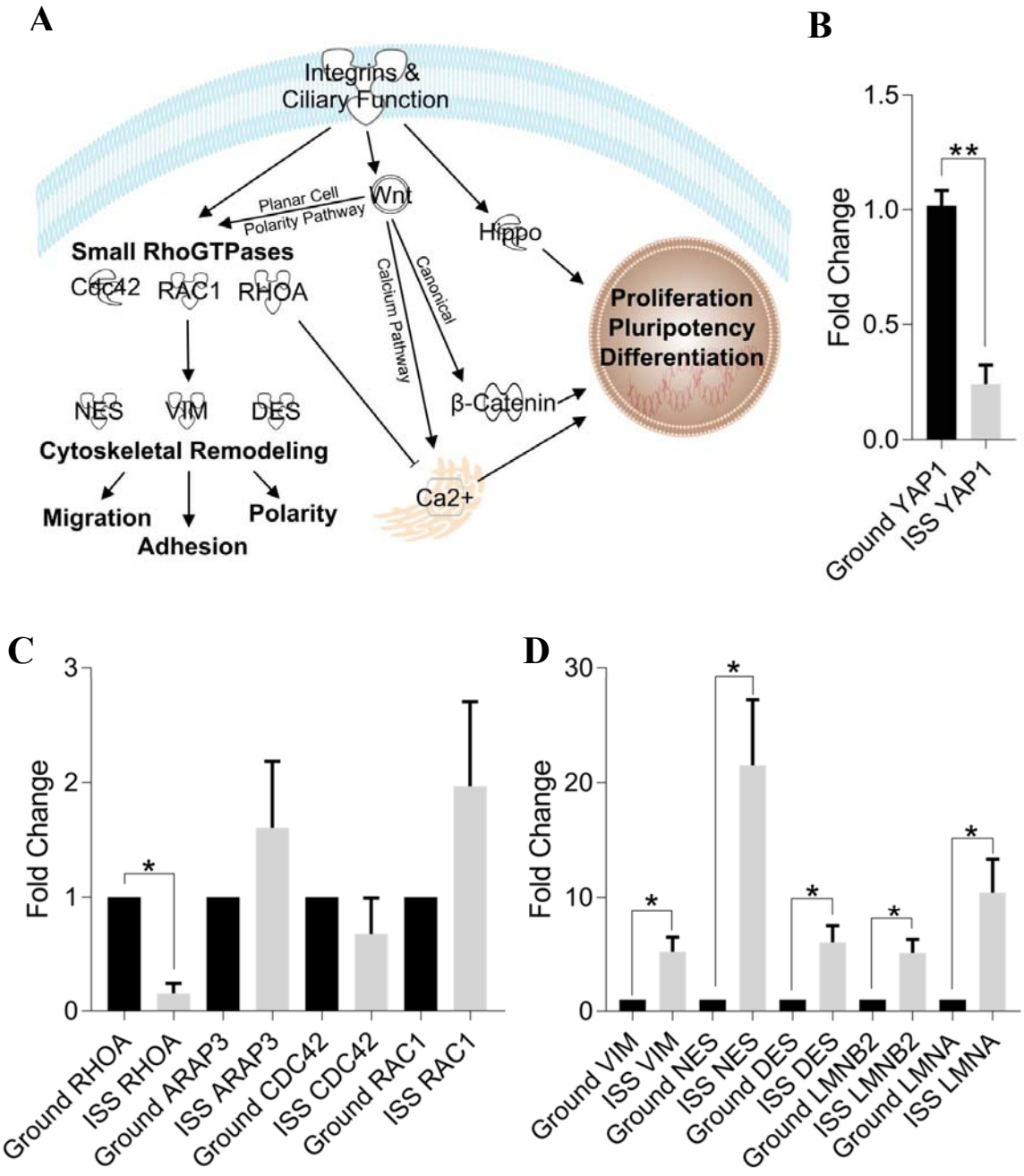


Figure 7. Microgravity impacts expression of genes involved in mechanotransduction and cytoskeleton maintenance in neonatal CPCs

Integrin and mechanical signaling impact Hippo activity and small RhoGTPases, which function along the Wnt planar cell polarity pathway (A). *YAP1* expression was significantly reduced in neonatal CPCs (B). *RHOA,* a small RhoGTPase, was expressed at significantly lower levels (C) CPCs. Accordingly, cytoskeletal gene expression was significantly upregulated (E). Data are reported as the mean ± SEM, n=3–6 per group, *p<0.05, **p<0.01.

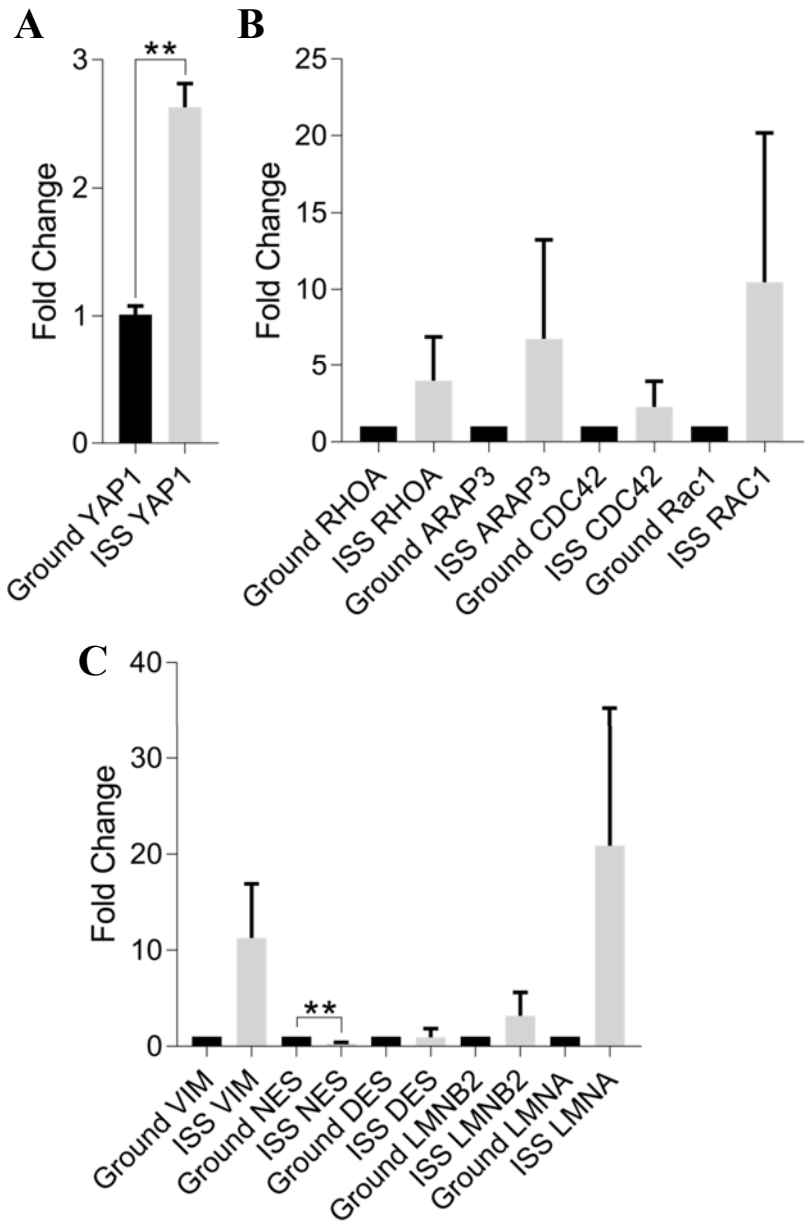


Figure 8. Microgravity generally does not impact the expression of genes involved in mechanotransduction and cytoskeleton maintenance in adult CPCs
YAP1 expression was significantly upregulated in adult CPCs (A). Small RhoGTPase gene (B) and cytoskeletal gene (C) expression was not significantly modulated by spaceflight. Data are reported as the mean ± SEM, n=3 per group, **p<0.01.

Microgravity Enhances Non-Canonical/Ca^{2+} Signaling Gene Expression in Neonatal CPCs

Given the role of small RhoGTPases in non-canonical, planar cell polarity Wnt signaling, we sought to assess the expression of genes involved in the calcium and canonical Wnt pathways. (Figs 9-10). In doing so, we observed significantly increased expression of *GSK3B* (fold-change: 6.798 ± 1.133, n=3 unique clones per group, P=0.036) in neonatal CPCs (Figure 9A) and significantly increased expression of *CTNNB1* (fold-change: 1.891 ± 0.121, n=3 unique clones per group, P=0.018) in adult CPCs (Figure 10A). Since Gsk3β sequesters β-catenin for degradation in the cytoplasm, thereby suggesting suppression of canonical Wnt signaling in neonatal CPCs and the promotion of canonical Wnt signaling in adult CPCs, we measured non-canonical Wnt ligand expression and that of genes in the Wnt/Ca^{2+} pathway. We measured significantly increased *WNT5A* and *WNT9A* expression (fold changes: 36.440 ± 8.258, P=0.050; 17.110 ± 3.411; P=0.042, respectively; n=3 unique clones per group) in neonatal CPCs (Figure 9B) and significantly decreased *WNT9A* expression (fold-change: 0.393 ± 0.170, n=3 unique clones per group, P=0.023) in adult CPCs (Figure 10B). This was supported by significantly increased expression of *PLCG1* and *PRKCA* (fold changes: 14.110 ± 1.468, P=0.012; 11.520 ± 2.974, P=0.0241; respectively; n=3 unique clones per group) and a general increase in *ITPR1* expression (fold changes: 2.668 ± 0.623, P=0.055; 10.240 ± 4.393; P=0.1034, respectively; n=3 unique clones per group) in neonatal (Figure 9C), but not adult (Figure 10C), CPCs.

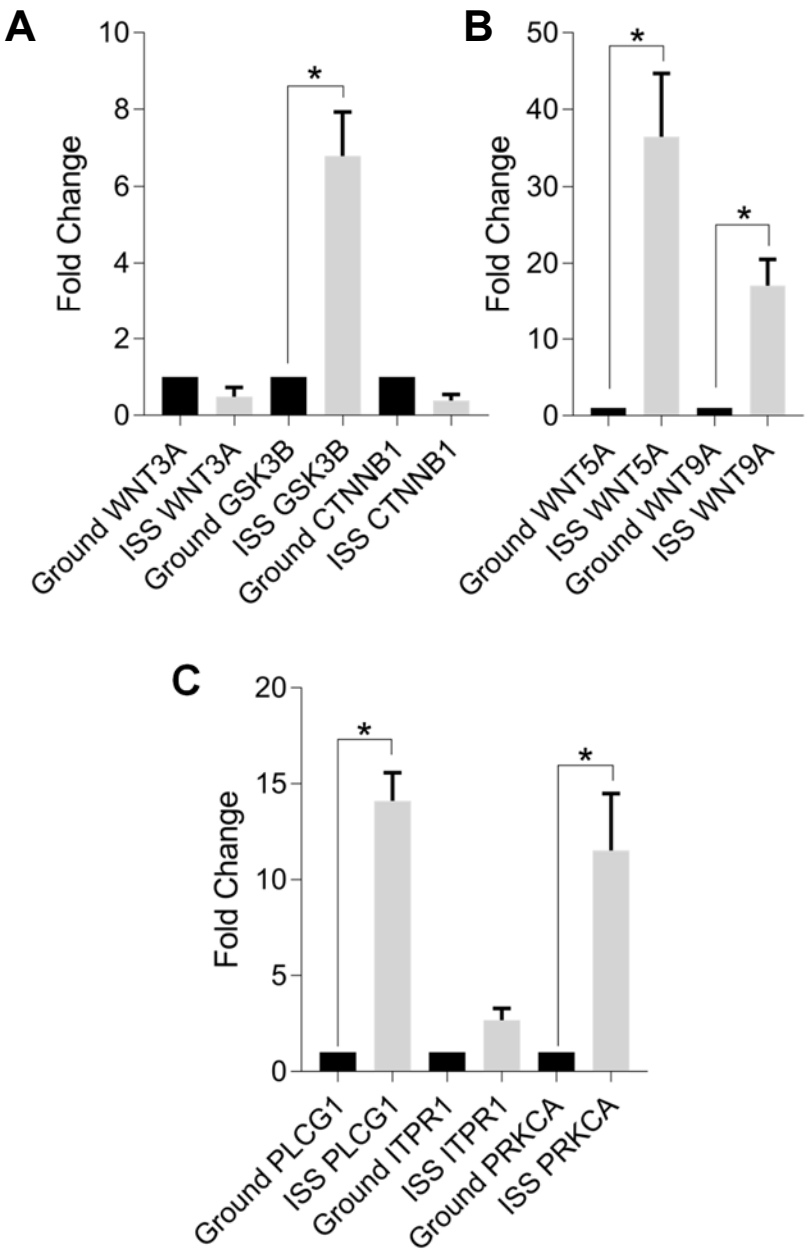


Figure 9. Genes involved in the non-canonical Wnt/Ca^{2+} pathway are expressed at higher levels in neonatal CPCs

Canonical Wnt signaling genes were measured in neonatal (A) CPCs along with non-canonical Wnt ligands (B). Genes involved in the non-canonical Wnt/calcium pathway were elevated in neonatal (C) CPCs. Data are reported as the mean ± SEM, n=3 per group, *$p<0.05$.

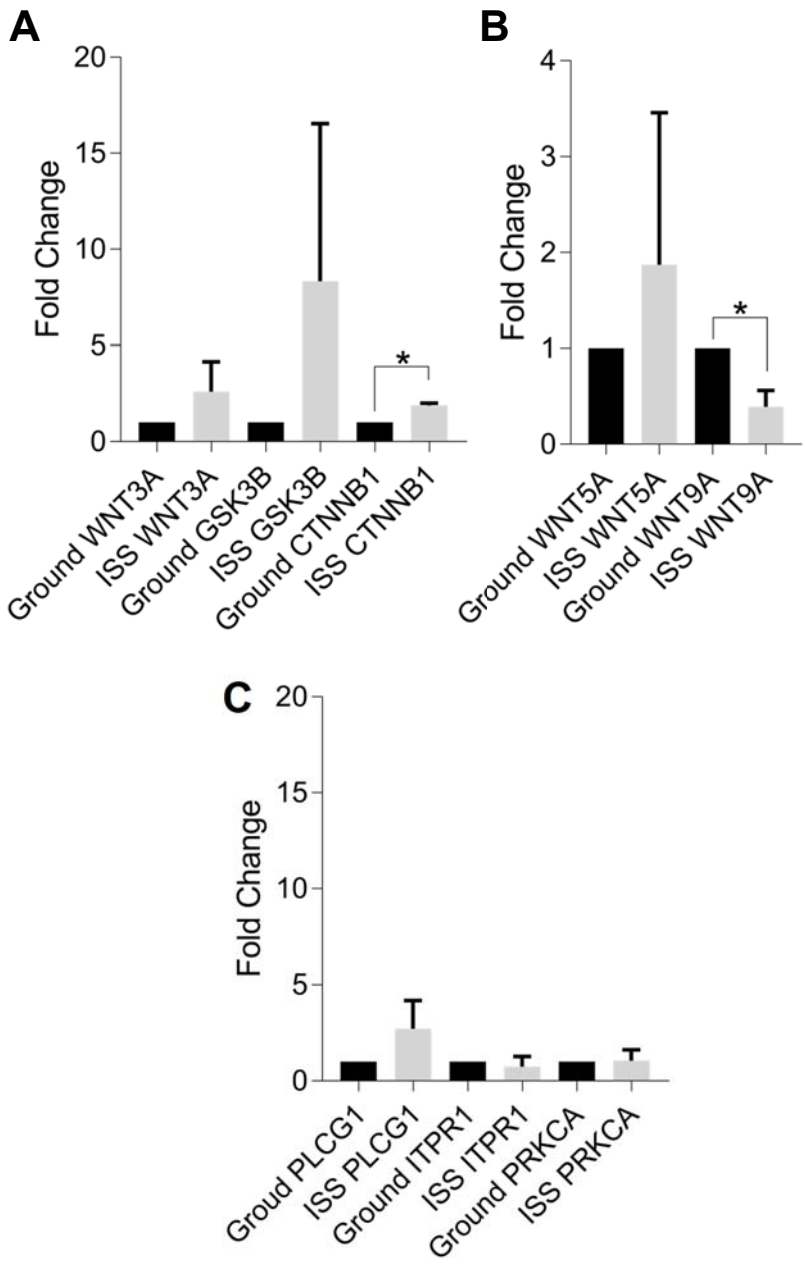


Figure 10. Genes involved in the non-canonical Wnt/Ca^{2+} pathway are not expressed at higher levels in adult CPCs

Canonical Wnt signaling genes were measured in adult (A) CPCs along with non-canonical Wnt ligands (B). Genes involved in the non-canonical Wnt/calcium pathway were unchanged in adult (C) CPCs. Data are reported as the mean ± SEM, n=3 per group, *$p<0.05$.

Neonatal CPCs Exhibit Increased Expression of Genes Involved in Pre-Mesodermal Development

Calcium and Wnt signaling have central roles in cardiogenesis. For this reason, we sought to assess the transcription of markers of various stages of cardiac development. We observed that the expression of genes involved in embryonic stem cell self-renewal (*POU5F1, NANOG, SOX2*), mesodermal specification (*FOXC1, MESP1, T*), and early cardiogenesis (*GATA4, KIT, PDGFRA, ISL1, KDR)* increased, while *NKX2-5* expression decreased, in neonatal CPCs (n=3 unique clones per group, Figure 11). In this population, the expression of genes involved in early mesodermal specification were the most significantly elevated. Conversely, adult CPCs generally exhibited decreased or unchanged expression of genes involved in early developmental processes, with the exception of *PDGFRA* (n=3 unique clones per group, Figure 11). Furthermore, increased telomerase activity, as indicated by enhanced *TERT* expression, was observed in neonatal CPCs following spaceflight (fold change: 6.782 ± 2.000, n=9 per group (three unique clones each measured in triplicate), P=0.020). Despite experiencing no change in expression in genes related to development, adult CPCs expressed higher levels of *TERT* expression (fold change: 21.150 ± 10.610, n=6 (three unique clones each measured in triplicate), P=0.007).

Expression of BMP4 and TBXs 3, 5, and 18 Is Elevated Under Microgravity in Neonatal CPCs

These modifications to the expression of genes involved in cardiogenesis motivated us to measure the changes in the expression of genes in the Bmp, Smad, and

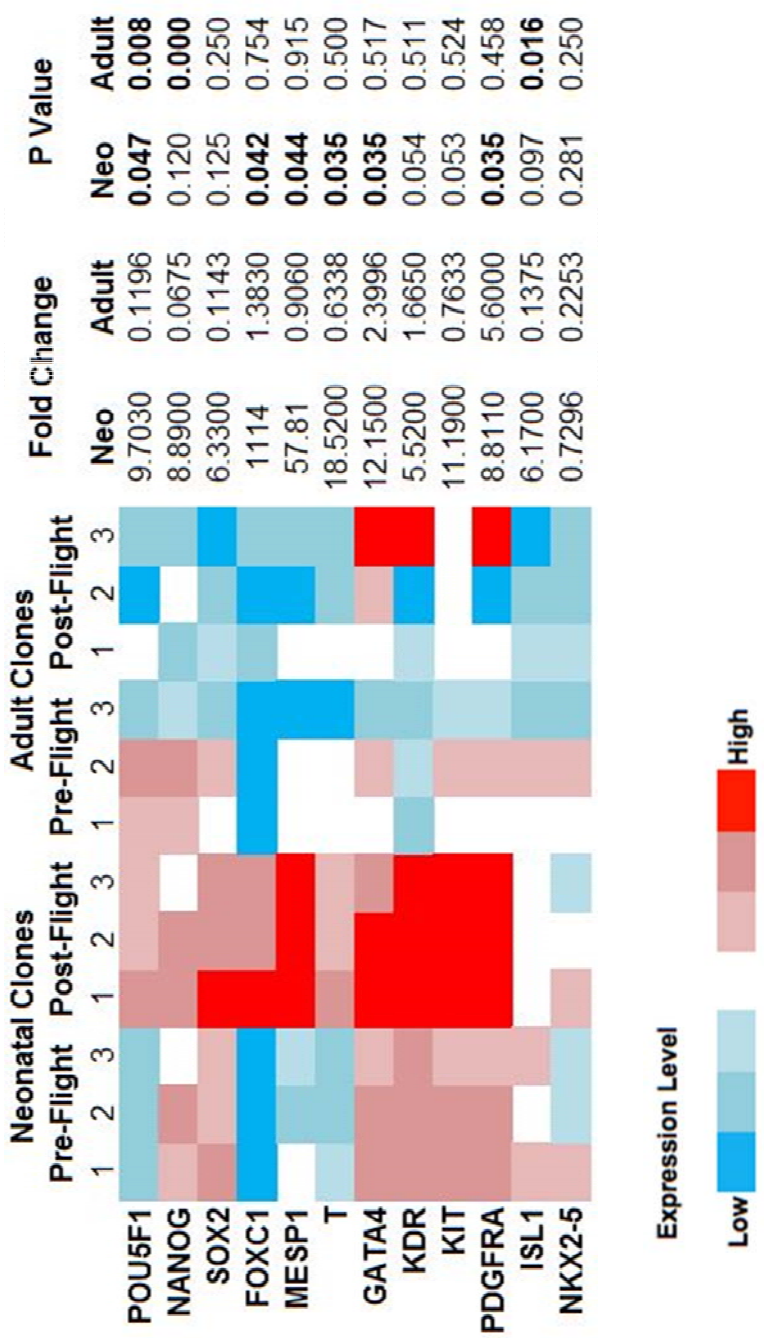


Figure 11. Spaceflight induces early mesodermal gene expression in neonatal CPCs Markers of development were measured in neonatal and adult CPCs following 12 days of culture aboard the International Space Station. Gene expression analysis indicated that early mesodermal markers (i.e., *FOXC1, MESP1,* and *T*) were elevated in expression in neonates, but not in adults. Data are reported as the mean. *p<0.05.

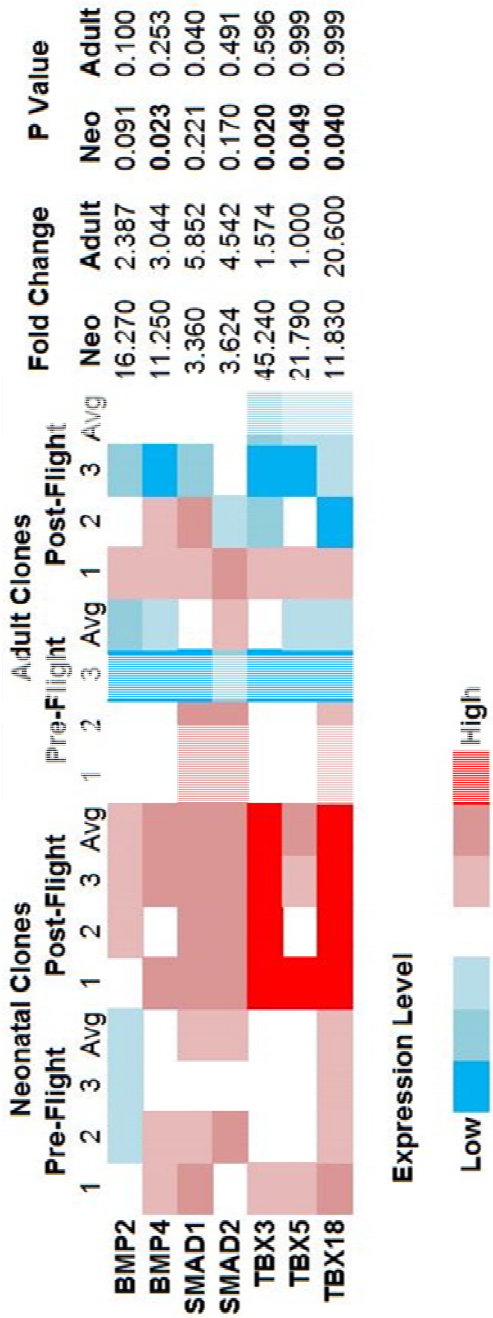


Figure 12. Spaceflight modifies TBX, Smad, and BMP expression patterns
The expression of members of the BMP, Smad, and TBX families of genes was measured after 12 days of culture aboard the ISS and was found to be elevated significantly in neonatal CPCs and modestly in adult CPCs. Data are reported as the mean.

Tbx families, which have important roles in pre- and early mesodermal development. We measured elevated expression of *BMP4, TBX3, TBX5*, and *TBX18*, and a general increase in *BMP2* expression in neonatal CPCs (Figure 12). In adult CPCs, *SMAD1* exhibited a significant increase in expression, while *SMAD2* and *TBX18* exhibited general increases in expression (Figure 12).

Microgravity Reduces Endothelial Tube Formation and Terminal Mesodermal Derivative Marker Expression in Neonatal CPCs

Given the elevated expression of early to pre-mesodermal genes in neonatal, but not adult, CPCs, we sought to measure whether MG enhances or reduces mesodermal derivative marker expression. Following 30 days in orbit, CPCs returned to Earth and were immediately assayed for endothelial tube formation capacity. Unlike adult CPCs, neonatal CPCs exhibited a significantly reduced ability to form tubes (n=3 measurements of four pooled clones per age per group, Figure 13). Representative images of tube formation of neonatal ground (Figure 13C) and ISS (Figure 13D) as well as adult ground (Figure 13E) and ISS (Figure 13F) samples reflect this age-dependent response of CPCs to spaceflight. Similarly, expression of the cardiomyocyte marker *TNNT2*, endothelial marker *VWF*, and osteoblast marker *RUNX2* were all significantly reduced in neonatal, but not adult, CPCs after 12 days in orbit. Interestingly, sinoatrial nodal markers *HCN4* and *SHOX2* exhibited elevated expression in neonatal CPCs and reduced expression in adult CPCs (n=3 unique clones per age per group, Figure 14).

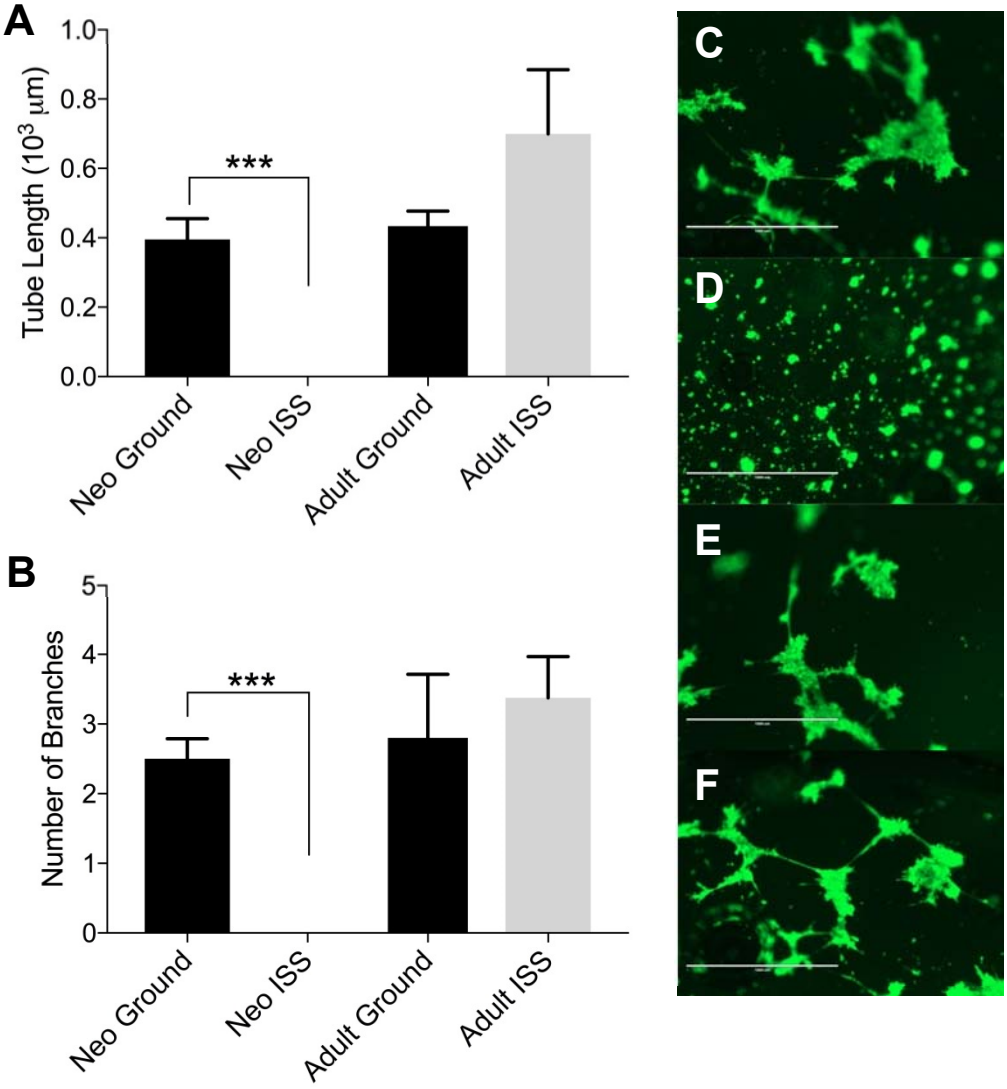


Figure 13. Spaceflight reduces the propensity for neonatal CPCs to readily form endothelial tubes

Following culture in biocells on the ground or the ISS for 30 days, CPCs were placed in endothelial growth media and incubated on Matrigel for 7 hours to facilitate endothelial tube formation. The area covered by the tubes that formed (A) and the number of branches (B) were quantified using ImageJ for neonatal ground (C) and ISS (D) CPCs as well as for adult ground (E) and ISS (F) CPCs (ruler = 1000µm). Data are reported as the mean ± SEM, n=3 measurements of four pooled clones, ***$p<0.001$.

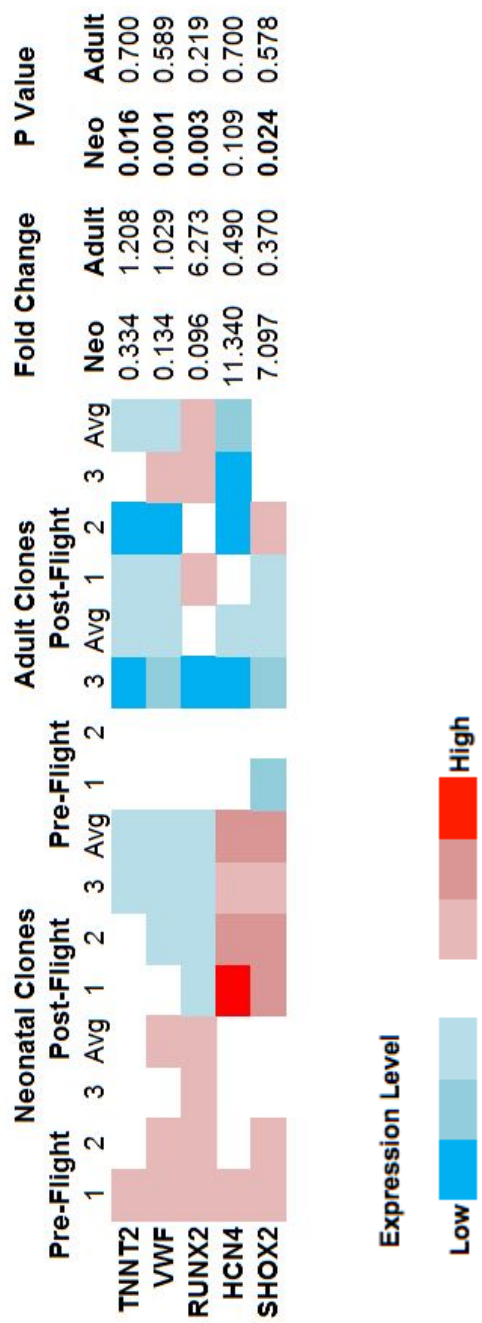


Figure 14. Spaceflight reduces the propensity for neonatal CPCs to express markers of terminal mesodermal derivatives

Changes in gene expression relevant to mesodermal derivatives were measured in CPCs cultured aboard the ISS for 12 days, indicating decreased expression of cardiomyocyte, endothelial, and osteogenic markers and increased expression of sinoatrial nodal markers. Data are reported as the mean ± SEM, ***p<0.001.

ISS-Cultured Neonatal CPCs Proliferate More Rapidly Than Adult or Ground-Control CPCs

To understand how these changes in early development marker expression affect proliferative potential, cell cycling was assessed using flow cytometry. The Dean-Jett-Fox model was fitted to fluorescence intensity curves generated for propidium iodide-stained neonatal and adult CPCs (n=3 measurements of four pooled clones per age per group; Figure 15A). Adult CPCs exhibited a significant decrease in the percentage of cells in the G1/G0 phase (ISS vs Ground: 70.9% vs 60.6%, p=0.003) and neonatal CPCs exhibited a significant increase in the percentage of cells in the G2/M phase (ISS vs Ground: 13.5% vs 17.17%, p=0.050; Figure 15B). Neonatal CPCs were more frequently in the G2/M phase both on the ground and following culture aboard the ISS. Interestingly, neonatal CPCs exhibited increases in *CDKN2A* (fold change: 14.080 ± 2.604, n=3 unique clones per group, p=0.037), *E2F1* (fold change: 9.012 ± 2.390, n=9 (three unique clones each measured in triplicate), p=0.010), and *PLK1* (8.509 ± 1.542, n=3 unique clones per group, p=0.040) (Figure 15C). These modulators of cell cycling were not significantly altered in adult CPCs (Figure 15D). Upon return to Earth, Biocells cultured aboard the ISS or on the ground contained 1,126,000 ± 176,000 or 152,300 ± 46,700 neonatal CPCs. Meanwhile, Biocells seeded with adult pools that were cultured aboard the ISS or on the ground contained 407,300 ± 92,700 or 246,000 ± 26,000 adult CPCs, respectively (Figure 15E). As expected from cell cycle data, the cell counts after 30 days in space were markedly larger in the neonatal CPC population (n=2 measurements of four pooled clones per age per group). Viability of CPCs upon return to Earth after 30 days of culture was not adversely affected (Figure 15F).

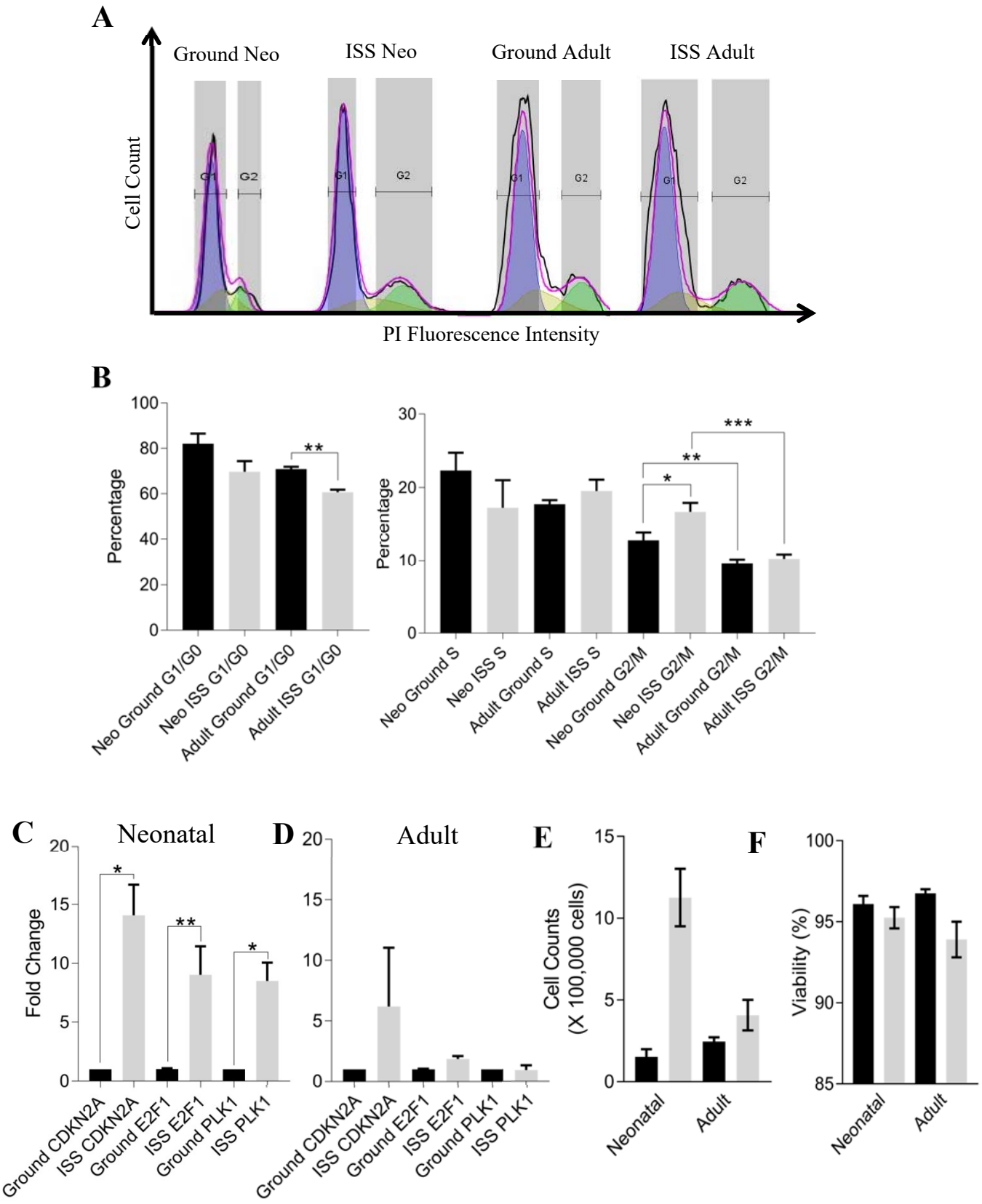

A
Ground Neo
ISS Neo
Ground Adult
ISS Adult
Cell Count
G1
G2
PI Fluorescence Intensity
B
Percentage
Neo Ground G1/G0
Neo ISS G1/G0
Adult Ground G1/G0
Adult ISS G1/G0
Neo Ground S
Neo ISS S
Adult Ground S
Adult ISS S
Neo Ground G2/M
Neo ISS G2/M
Adult Ground G2/M
Adult ISS G2/M
C
Neonatal
Fold Change
Ground CDKN2A
ISS CDKN2A
Ground E2F1
ISS E2F1
Ground PLK1
ISS PLK1
D
Adult
E
Cell Counts (X 100,000 cells)
Neonatal
Adult
F
Viability (%)

Figure 15. Cell cycling and proliferation are enhanced following spaceflight in neonatal CPCs

Following 30 days of culture aboard the ISS, CPCs were fixed, stained with propidium iodide, and measured using flow cytometry. The Dean-Jett-Fox model was then applied to the histogram of propidium iodide fluorescence intensity for ground- and ISS-cultured CPCs (A). Upon analysis (B), adult CPCs exhibited a significant decrease in the percentage of cells in the G1/G0 and neonatal CPCs exhibited a significant increase in the percentage of cells in the G2/M phase. This was supported by increased expression of *CDKN2A, E2F1,* and *PLK1*, which function to regulate G1/S arrest, overcome G1/S arrests, and promote G2/M progression, respectively, in neonatal (C), but not adult (D), CPCs. Despite enhanced proliferation (E), viability was not adversely affected (F). Data are reported as the mean ± SEM, n=3 measurements of four pooled clones per group for cell cycling analysis; n=3–9 per group for all gene expression data, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

DNA Repair Transcripts Increased Following Spaceflight

The broad alterations experienced by CPCs and the possibility of exposure to radiation in the extraterrestrial environment prompted us to consider possible cellular stress response mechanisms, including the expression of DNA repair gene programs. We measured increased expression of genes involved in DNA repair, as indicated by elevated levels of *ATM* (fold change: 10.852 ± 3.446, n=9 (three unique clones each measured in triplicate), P=0.021), *RAD23* (fold change: 7.943 ± 0.789, n=9 (three unique clones each measured in triplicate), P<0.001), and *RAD50* (fold change: 21.250 ± 8.656, n=9 (three unique clones each measured in triplicate), P=0.033) (Figure 16A). Adult CPCs generally exhibited increased expression of DNA repair genes (Figure 16B); however, only *ATM* (fold change: 23.910 ± 0.682, n=9 (three unique clones each measured in triplicate), P=0.001) expression was significantly elevated.

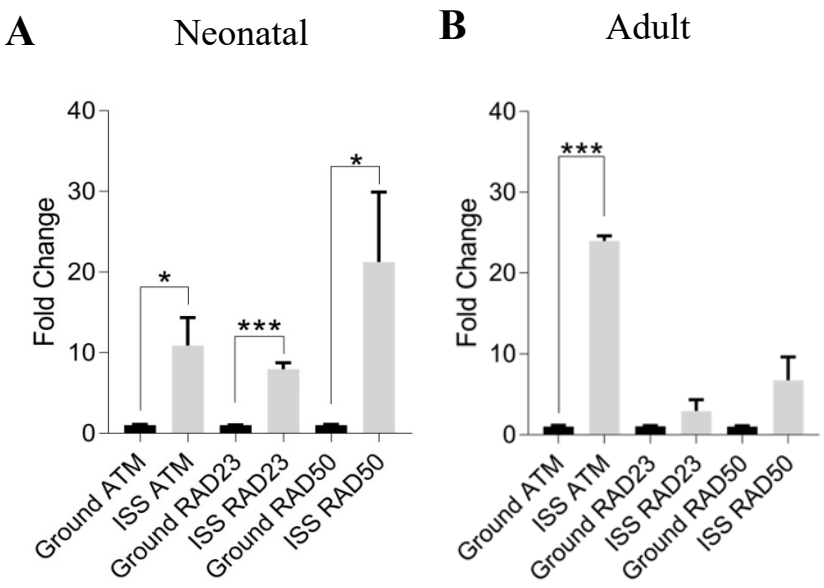


Figure 16. DNA repair genes are induced by spaceflight in an age-independent manner.

DNA repair gene expression was elevated, as indicated by increased levels of *ATM, RAD23,* and *RAD50* expression (A). Adult CPCs generally exhibited increased DNA repair genes; however, only *ATM* expression was significantly increased (B). Data are reported as the mean ± SEM, n=6–9 per group, *p<0.05, **p<0.01, ***p<0.001.

Stress Response, But Not Apoptosis, Genes Are Induced in CPCs

Given the broad induction of transcripts associated with DNA repair, we sought to characterize the impact of other stressors that could influence cellular function during spaceflight. Neonatal CPCs exhibited a 25-fold increase ($P<0.05$) in *NOTCH1* expression (Figure 17A-B), which confers protection as part of the adaptive cardiac stress response (Croquelois, 2008). In considering oxidative stress and that associated with spaceflight (Figure 17C-D; Cubano, 2001; Versari, 2013; Zupanska, 2013), we found that *HSP70* was elevated in both neonatal (12-fold, $P<0.01$) and adult (20-fold, $P<0.05$) CPCs, while *TXNIP*, *TP53INP*, and *HMOX1* were all induced only in adult CPCs (21-fold, $P<0.01$; 2.2-fold, $P<0.05$; 3.2-fold, $P<0.001$; respectively). Ultimately, neither adult not neonatal CPCs exhibited an induction of transcripts associated with the apoptosis pathway (Figure 17E-F). As discussed previously, neither proliferation nor viability was reduced in either age group.

Adult and Neonatal CPCs Both Exhibit a Greater Migratory Capacity after ISS Culture

In addition to changes in the regulation of differentiation, cytoskeletal alterations were anticipated to occur in response to MG. For this reason, we measured the migratory capacity of ISS-cultured CPCs using a Transwell migration assay. Both neonatal and adult CPCs that were cultured aboard the ISS migrated at significantly higher levels in response to SDF-1α stimulation (Neonate (ISS vs Ground): 2963 ± 15 cells vs 1972 ± 69 cells; n=5 measurements of four pooled clones per group; $P=0.008$; Adult (ISS v

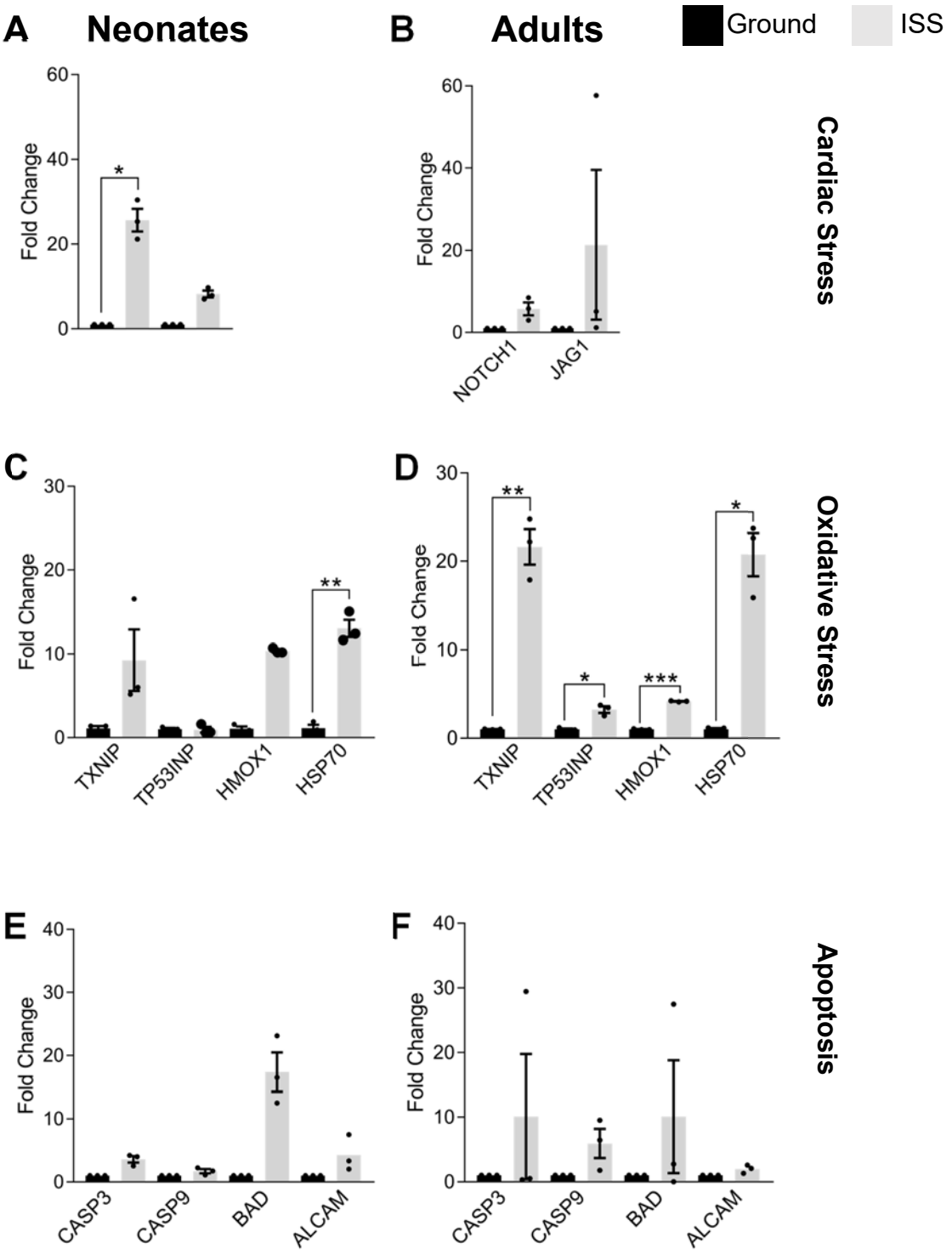

A Neonates
B Adults
Ground
ISS
Cardiac Stress
Oxidative Stress
Apoptosis
Fold Change
NOTCH1
JAG1
TXNIP
TP53INP
HMOX1
HSP70
CASP3
CASP9
BAD
ALCAM

Figure 17. CPCs exhibit minimal induction of genes involved in stress response pathways

Given the harsh nature of the extraterrestrial environment, we sought to assess the impact of spaceflight on stress pathways. We measured changes in gene expression pertinent to the cardiac (neonatal, A; adult, B) and oxidative (neonatal, C; adult, D) stress response as well as apoptosis activation (neonatal, E; adult, F) in adult and neonatal CPCs flown aboard the ISS for 12 days. n=3 biological replicates. Data: mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

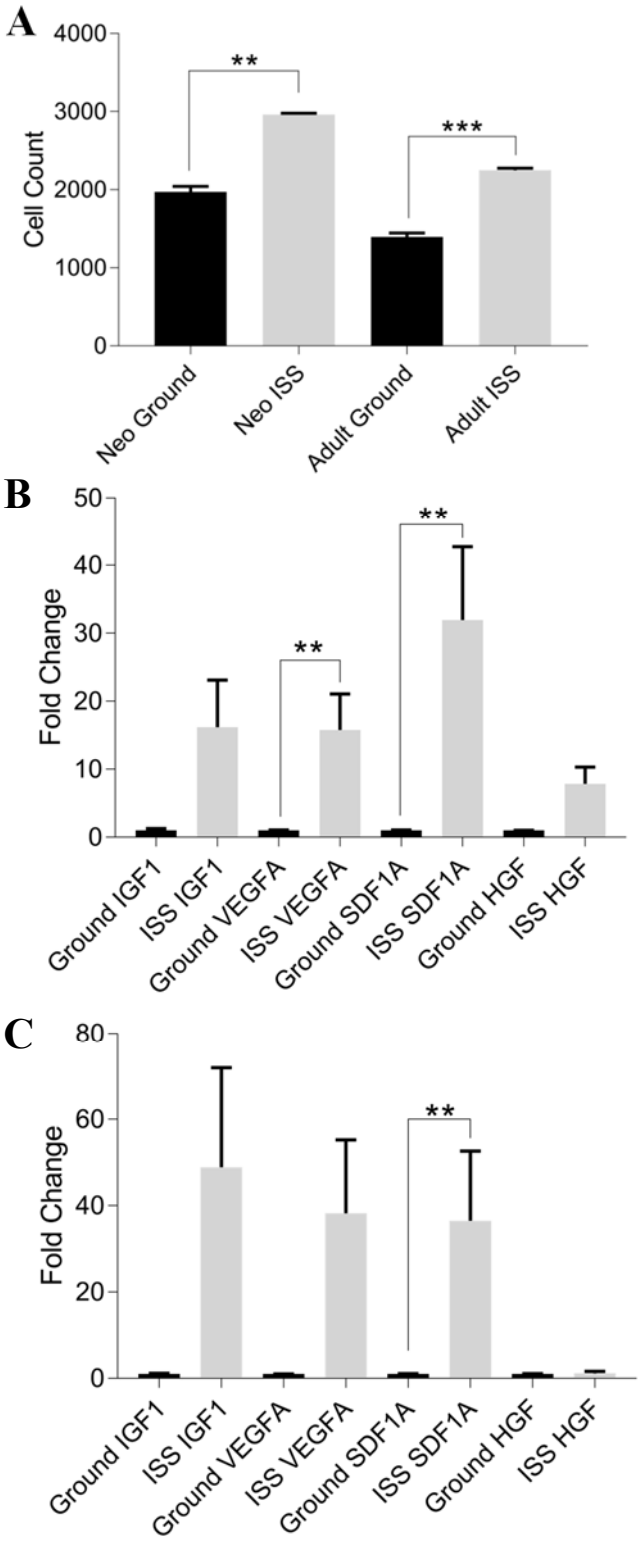

A
4000
3000
2000
1000
0
Cell Count
**
***
Neo Ground
Neo ISS
Adult Ground
Adult ISS
B
50
40
30
20
10
0
Fold Change
**
**
Ground IGF1
ISS IGF1
Ground VEGFA
ISS VEGFA
Ground SDF1A
ISS SDF1A
Ground HGF
ISS HGF
C
80
60
40
20
0
Fold Change
**
Ground IGF1
ISS IGF1
Ground VEGFA
ISS VEGFA
Ground SDF1A
ISS SDF1A
Ground HGF
ISS HGF

Figure 18. CPCs have enhanced migratory capacity following spaceflight
Both neonatal and adult CPCs that were cultured aboard the ISS migrated at significantly higher levels in response to SDF-1α stimulation (A). Similarly, *SDF1A* expression was elevated in both neonatal (B) and adult (C) CPCs following exposure to low Earth orbit. *VEGFA* expression was also significantly elevated in neonatal CPCs (B), while most other paracrine and growth factors generally increased regardless of age. Data are reported as the mean ± SEM, n=6–9 per group, **$p<0.01$, ***$p<0.001$.

Ground): 2247 ± 28 cells vs 1394 ± 52 cells; n=5 measurements of four pooled clones per group; P<0.001) (Figure 18A). Similarly, *SDF1A* transcripts were elevated in both neonatal (Figure 18B) and adult (Figure 18C) CPCs following exposure to low Earth orbit (Neonate: 31.950 ± 10.770-fold increase, n=9 (three unique clones each measured in triplicate), P=0.0039; Adult: 36.480 ± 16.150-fold increase, n=9 (three unique clones each measured in triplicate), P=0.0078). *VEGFA* transcripts were also significantly elevated in neonatal CPCs (15.78 ± 5.283-fold increase; n=9 (three unique clones each measured in triplicate); P=0.0068).

Discussion

In this study, we found that microgravity exerts broad effects on the developmental status, proliferative potential, and migratory ability of CPCs, some of which occurred in an age-dependent manner. In particular, neonatal CPCs exhibited increased expression of early developmental markers, enhanced proliferative potential, and increased migratory capacity under MG. Meanwhile, adult CPCs experienced little change in the expression of genes indicative of their developmental state, but exhibited changes to migratory capacity. These findings suggest that broad cytoskeletal modifications resulting from reduced mechanotransduction impart improved migratory and adhesion capabilities regardless of age, but that neonatal CPCs are able to propagate or experience additional intracellular signaling events (e.g., calcium signaling) that modify their developmental status. As we have shown elsewhere, one important difference between adult and neonatal CPCs is their epigenetic (i.e., microRNA) environment (Fuentes, 2013).

Several microRNAs were observed to be differentially regulated in response to spaceflight in neonatal and adult CPCs. Interestingly, the predicted targets of these microRNAs included activators and repressors of several pathways. A detailed inspection of the putative targets under each pathway impacted by the microRNAs identified in this study revealed the targeting of genes relating to Wnt signaling (*FZD3*, *FZD5*, *FZD7*, *FZD8*), cytoskeletal regulation (*ACTB, ACTG1, LAMC3*), cardiovascular development (*TGFBR1*, *BMP2*, *BMP5*, *SMAD7*), integrin function (*ITGB4*, *ITGAV*), extracellular proteins (*COL4A2, COL5A1, COL1A1, COL1A2, COL4A1)*, and Hippo signaling (*TEAD1, YAP1, LATS1*). Interestingly, miR-16, whose expression was generally elevated in neonatal CPCs and repressed in adult CPCs, directly targets *YAP1*, which could account, at least in part, for the age-dependent response of CPCs to spaceflight. However, since KEGG analysis only identifies broad categories of genes (i.e., functional categories) that are likely targets of the microRNAs under study, further experiments are warranted to identify the cumulative impact of these microRNAs on signaling in space flown CPCs.

These results motivated us to assess the overall impact of spaceflight on *YAP1*, an effector of Hippo signaling that functions with TAZ to regulate transcription. Activation of Hippo kinases, such as through a loss of microRNA-mediated repression or through reduced mechanical signaling, results in elevated levels of phosphorylated Yap1 and Taz. This prevents translocation of these transcriptional activators to the nucleus. Similarly, the application of SMG to bone mesenchymal stem cells prevented TAZ translocation to the nucleus, thereby preventing the expression of *RUNX2* and inhibiting osteogenesis (Chen, 2016). Similarly, the decreased expression of *YAP1* and *RUNX2* observed in this experiment indicate that spaceflight reduces mechanotransduction in neonatal CPCs.

In addition to broadly targeting the Hippo signaling pathway and ECM interactions, select microRNAs that were modified by MG are known to be critically involved in cardiac development. For example, miR-142-3p is involved in targeting genes involved in early cardiac development, such as *TBX5* (Chen, 2017). Meanwhile, miR-302, which targets the type II BMP receptor to regulate early cardiogenic events, is repressed by Bmp4 in a Smad-dependent manner (Kang, 2012). Interestingly, we observed both generally reduced expression of miR-302c and a generally elevated expression of *BMP4* in CPCs exposed to MG. Moreover, studies have demonstrated a direct mechanistic role of certain microRNAs in cardiac regeneration. As neonatal mice age, their cardiomyocytes lose their proliferative potential in a manner that is concomitant with increased let-7 family expression (Porrello, 2011), thereby indicating a relationship between let-7 over-expression and reduced proliferative potential. Indeed, let-7c along with miR-99/100, which were significantly down-regulated in neonatal CPCs, are also critically down-regulated during cardiomyocyte proliferation in a zebrafish model after ventricle amputation (Aguirre, 2014). As part of the amputation injury response, zebrafish cardiomyocytes near the site of injury undergo sarcomeric structure disassembly as a form of limited de-differentiation to prepare myocytes for self-renewal. *PLK1*, which was upregulated in CPCs cultured aboard the ISS, is involved in this process (Jopling, 2010). Interestingly, these changes to the cytoarchitecture of cardiomyocytes and subsequent limited de-differentiation appear to resemble the effects of SMG (Fuentes, 2013) and MG on CPCs.

Our laboratory has previously measured changes in microRNA expression in neonatal CPCs induced by SMG (Fuentes, 2013). In comparing those findings to the

microRNA changes induced by culture aboard the ISS, we found miR-99a and -100 as well as members of the let-7 family, all of which are implicated in differentiation and developmental regulation, exhibit significantly reduced expression under both conditions. Elsewhere, we have compared the effects of spaceflight and SMG on the induction of signaling in neonatal CCPs and observed similar activation of calcium and Akt signaling. When considered together these findings suggest that reduced gravity conditions readily alter the developmental properties of neonatal CPCs, possibly through reduced mechanotransduction (Baio, 2018).

Alterations to microRNA expression as well as changes to ECM interactions in MG-treated CPCs highlight the significance of the environment in directing differentiation. For example, a single population of mesenchymal stem cells can express neurogenic, myogenic, or osteogenic transcription factors when cultured on soft, moderately stiff, or stiff matrices, respectively (Engler, 2006). In our experiments, we observed elevated expression of mesendodermal-stage developmental markers in neonatal CPCs. It is likely that ECM interactions prompted cytoskeleton remodeling while modifying intracellular signaling pathways such that proliferation and stemness were enhanced. Similar experiments in different cell types involving SMG or a low Earth orbit cell culture system have demonstrated that reduced gravity conditions modify the differentiation potential of stem cells. Mouse ESCs flown as embryoid bodies during the Space Tissue Loss Experiment exhibited a decrease in the expression of markers of terminal germ layer derivatives while maintaining expression of stem cell renewal markers (Blaber, 2015). However, upon return to Earth, these mouse ESCs differentiated more readily into colonies of contracting cardiomyocytes. Mesenchymal stem cells

cultured under conditions of SMG exhibit a reduction in the expression of markers of cartilage and osteoblast formation (Yuge, 2006; Dai, 2007), while also demonstrating enhanced proliferation potential and the maintenance or enhancement of stemness under SMG (Yuge, 2006; Zhang, 2015).

In addition to the expression of genes that are indicative of an earlier stage of development, neonatal CPCs exhibited both a reduced capacity to readily form endothelial tubes and decreased expression of mesodermal derivative markers (i.e., cardiomyocte, endothelium, and osteoblast) under MG.

This concomitant induction of mesendodermal markers and Tbx gene expression reflects the potential therapeutic value of the MG environment for cardiac repair. The critical role of the Tbx family in cardiac development has been well documented (Plageman, 2005) and its application to cellular therapies for cardiac repair is beginning to be explored. For example, SSEA-1^{+} early cardiovascular progenitors expressing *T, TBX5*, and *TBX18*, were shown to engraft into non-human primate myocardium following infarction (Blin, 2010). Meanwhile, *Tbx5* and *Tbx18* are expressed in highly migratory and proliferative proepicardial cell lineages (Tanaka, 2004; Hatcher, 2001). These studies have demonstrated an increasingly appreciated observation that Tbx expression is related to the engraftment and function of CPCs following transplantation.

Beyond directing developmental events via alterations in intracellular signaling, disrupted cytoskeletal organization impacts migration. In other models, cytoskeletal re-organization induces the expression of *CXCR4,* the SDF-1α chemokine receptor (Lin, 2016). As seen in our experiments, both neonatal and adult CPCs exhibited elevated

expression of *SDF1A,* which correlates to the age-independent increase in migratory capacity of CPCs following spaceflight.

Interestingly, the age-dependent changes in developmental status following culture aboard the ISS suggest fundamental differences in adult CPCs. While KEGG analysis demonstrated that functionally similar pathways are likely affected in both adult and neonatal CPCs, only the latter population experienced significant changes to the expression of cytoskeletal genes and early developmental markers. This trend was supported by the significantly increased cell cycle and proliferation patterns observed only in neonatal CPCs. Broad differences have already been reported to exist between adult and neonatal CPCs, including at the genetic/epigenetic (Fuentes, 2013) and proteomic levels (Sharma, 2017). In particular, the secretome of adult CPCs was found to exhibit a greater number of proteins related to anti-proliferation, pro-apoptosis, and senescence. Meanwhile, the secretome of neonatal CPCs exhibited higher levels of telomerases as well as proteins related to cell cycle, proliferation, and anti-apoptosis (Sharma, 2017). Similarly, we observed age-disparate responses to spaceflight in nearly all of these categories. In the context of this experiment, microRNA dysregulation may have exerted a greater effect on neonatal CPCs given their relatively higher levels of expression of genes related to these processes. Nevertheless, the prospective use of CPCs in autologous cardiac stem cell therapies in a predominately adult population requires that these functional differences be elucidated.

As humans prepare to expand our presence in space, it is imperative to deepen our understanding of the nature of cellular adaptation to reduced gravity so that we may develop mechanisms by which these molecular changes in cardiac cell types can be

countered. Meanwhile, on Earth, we can further explore the therapeutic potential of these adaptations for cardiac repair.

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CHAPTER THREE

PROTEIN KINASE C ALPHA SIGNALING IS ACTIVATED BY REDUCED GRAVITY CONDITIONS AND VIA TREATMENT WITH WNT5A ON EARTH

This chapter has been previously published in:

Baio JM, Martinez AF, Bailey L, Hasaniya N, Pecaut MJ, Kearns-Jonker M. Spaceflight Activates Protein Kinase C Alpha Signaling and Modifies the Developmental Stage of Human Neonatal Cardiovascular Progenitor Cells. Stem Cells and Development. In Press.

Abstract

As demonstrated in chapter two, neonatal cardiovascular progenitor cells (CPCs) experience alterations to gene expression and function in response to reduced gravity conditions. Furthermore, culture of neonatal CPCs aboard the International Space Station (ISS) was observed to promote migration and foster proliferation. Thus, the potential therapeutic benefit of culturing stem cells under conditions of simulated microgravity or spaceflight merit identifying the molecular events underpinning CPC adaptation to space. Therefore, following 12 and 30 days of culture aboard the ISS, neonatal CPCs were assessed for changes in gene expression and signaling activation. In doing so, we observed that ISS-cultured CPCs exhibited elevated levels of calcium handling and signaling genes, which corresponded to protein kinase C alpha (PKCα), a calcium-dependent protein kinase, activation after 30 days. Additionally, Akt was activated, whereas phosphorylated Erk levels were unchanged. To explore the effect of calcium induction in neonatal CPCs, we activated protein kinase C alpha (PKCα) using hWnt5a treatment on Earth. Subsequently, early cardiovascular developmental marker levels were elevated. These findings demonstrate that calcium signaling is both sensitive to reduced gravity conditions and involved in directing the developmental profile of early cardiovascular progenitor cells.

Introduction

The effects of spaceflight on cardiovascular progenitor cells (CPCs) critically impacts processes related to development and mechanotransduction via extracellular matrix interactions. Such findings relate to our observations under simulated microgravity, in which earlier developmental genes (Fuentes, 2015) and genes involved in non-canonical Wnt/Ca^{2+} signaling (Baio, 2018) were induced. Similar research that exposed neural crest progenitors to simulated microgravity observed increased *AXIN2* and *GSK3β* expression, whose protein products inhibit canonical Wnt signaling (Hatzistergos, 2018). In addition to the induction of non-canonical Wnt ligands, small RhoGTPases, such as RhoA, are intimately involved in calcium potentiation or inhibition (Kim, 2009). Thus, the more broadly observed involvement of RhoA in stem cell responses to simulated microgravity (Meyers 2005; Pan, 2007; Seki, 2006) likely implicate alterations to calcium activity *in vivo*. In the context of cardiogenesis, studies performed in both embryos and ESC-derived cardiomyocytes have shown a critical role of Ca^{2+} in regulating multiple steps of heart formation (Pucéat, 2005). Meanwhile, the modification of signaling pathways related to calcium, either as an important secondary or mediating molecule, directly impacts the ability of CPCs to differentiate or maintain pluripotency (Tonelli, 2012; Apati, 2016). Thus, we speculated that calcium activity may be intimately related to the response of early CPCs to reduced gravity conditions.

For this reason, we sought to elucidate the role of calcium signaling in the response of CPCs to spaceflight and to identify peripheral pathways that may be involved in this process. We also sought to extend these space-borne observations to *in vitro* experiments on Earth.

Materials and Methods

Ethics Statement/Cell Isolation and Expansion

The Institutional Review Board of Loma Linda University approved the protocol for use of tissue that was discarded during cardiovascular surgery, without identifiable private information, for this study with a waiver of informed consent. CPCs were isolated from cardiac tissue of neonates (1 day – 1 month), as previously described (Fuentes, 2013). Briefly, atrial tissue was cut into small clumps (approximately 1.0 mm^3) then enzymatically digested using collagenase (Roche, Indianapolis, IN) at a working concentration of 1.0 mg/mL. The resulting solution was then passed through a 40-µm cell strainer. Cells were cloned in a 96-well plate by limiting dilution to a final concentration of 0.8 cells per well to create populations for expansion. Then, clones were screened for the co-expression of Isl1 and c-Kit and supplemented with growth media comprised of 10% fetal bovine serum (Thermo Scientific, Waltham, MA), 100 µg/mL penicillin-streptomycin (Life Technologies, Carlsbad, CA), 1.0% minimum essential medium non-essential amino acids solution (Life Technologies, Carlsbad, CA), and 22% endothelial cell growth media (Lonza, Basel, Switzerland) in Medium 199 (Life Technologies, Carlsbad, CA). The MycoAlert PLUS Mycoplasma Detection Kit (Lonza, Basel, Switzerland) was used to test for mycoplasma contamination.

Progenitor cell populations were fluorescently labeled with antibodies, as recommended by their respective manufacturers, and then analyzed using a MACSQuant® analyzer (Miltenyi Biotec, Auburn, CA). Quantification of data was performed using FlowJo software version 10 (Ashland, OR). Isotype controls and unstained cell populations

were used to define positive and negative gates. Antibodies used for cytometric analysis are described in Table 3.

Biocell Seeding and Spaceflight

For experiments described herein, neonatal CPCs were seeded into 8 polystyrene Biocells (BioServe Space Technologies, Boulder, CO) containing 20 mL of growth media. Biocells were loaded into self-contained environments containing 5% CO_2 and 95% air, and flown aboard SpaceX CRS-11 to the US National Lab on the ISS. Three days after launch, the Biocells arrived at the national lab, where they received fresh growth media and were placed in an incubator containing 5% CO_2 and 95% air. Thereafter, fresh media was provided every four to five days while aboard the ISS (Figure 3). Biocells were seeded with CPCs derived from four unique neonates (1 day to 1 month) before being fixed at 12 days after arrival to the ISS with RNA Protect (Qiagen, Valencia, CA) and stored at -80ºC or fed and returned to Earth at 30 days after arrival to the ISS for immediate processing. Clone- and passage-matched ground controls were fed and treated in parallel with the feeding schedule and activities performed by our astronaut collaborator aboard the ISS.

Post-Flight Sample Processing

Upon landing and retrieval of the payload, live cells were trypsinized, counted, and used to generate protein lysates. Biocells containing cells fixed and frozen in RNA protect were thawed at room temperature. The RNA protect was removed and centrifuged at 10,000g at 4ºC for 10 minutes. Biocells were disassembled and the culture membranes

were then rinsed with TRIzol® reagent (Life Technologies, Carlsbad, CA). RNA was purified from RNA protect samples using the RNeasy Mini Kit (Qiagen, Valencia, CA), per the manufacturer's instructions, while total RNA was purified from TRIzol® reagent using isopropanol- and ethanol-based precipitation. cDNA was generated and RT-PCR was performed as described below.

Gene Expression Profiler Array

We used custom array plates (CLAH22469A; Qiagen, Valencia, CA) per the manufacturer's instructions to measure gene expression changes in ISS-cultured adult and neonatal CPCs that were relevant to Wnt, ERK, BMP/Smad, and Notch signaling; cytoskeletal maintenance; calcium handling; apoptosis and cell cycle; cardiac development and stemness; and regeneration. Briefly, 2 μg of RNA was reverse transcribed into cDNA, as described above, and then thoroughly mixed with 2X RT^2 SYBR Green Mastermix and RNase- and DNase-free water before being loaded into the profiler array plate. Samples were amplified in the iCycler iQ™5 PCR Thermal Cycler (Bio-Rad, Hercules, CA) using a protocol of 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for one minute. Fold changes were determined for each clone individually using the Qiagen Data Analysis Center (http://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/) using *GAPDH* and *ACTB* (β-actin) as housekeeping genes. Since this analysis center performs only a two-tailed Student's t-test to calculate P-values, all fold changes for individual clones were exported to Prism and analyzed, as described below.

Quantitative RT-PCR

cDNA was prepared using 2 µg of RNA with Superscript III (Life Technologies, Carlsbad, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Go-Taq® qPCR Mastermix (Promega, Madison, WI) and the iCycler iQ™5 PCR Thermal Cycler (Bio-Rad, Hercules, CA) following a protocol of 94°C for 10 minutes followed by 45 cycles of 94°C for 15 seconds, 52–68°C (depending on the primer) for 60 seconds, and 72°C for 30 seconds. RT-PCR products were visualized using 1–2% agarose gel electrophoresis and low mass DNA ladder (Invitrogen, Carlsbad, CA). Primers were designed using the National Center for Biotechnology Information Primer-BLAST program and obtained from Integrated DNA Technologies (Coralville, IA). Primers used in experiments are listed in Table 7.

Protein Simple-Based Western Blotting

Following detachment, CPCs were homogenized using RIPA buffer containing phosphatase inhibitor cocktail (Millipore, Temecula, CA), followed by centrifugation at 14,000 g for 15 minutes at 4°C and collection of the supernatant for analysis. Total protein concentrations were determined using the Pierce Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). A capillary-based western blotting system (ProteinSimple Wes, San Jose, CA) was used to assess protein expression. All procedures were completed according to the manufacturer's instructions and default settings. The concentration of protein lysates, antibodies used in experiments, and antibody dilutions

Table 7. Primer pairs used in RT-PCR for ISS experiments (5' to 3')

Gene	Forward Sequence	Reverse Sequence
ACTIN	TTTGAATGATGAGCCTTCGTCCCC	GTCTCAAGTCAGTGTACAGGTAAGC
CAMK2A	GTCCAGTTCCAGCGTTCAGTT	GTGGGGATTCAGGATGGTGG
CCND1	TGCTGGAGTCAAGCCTGCGC	AGGACATGCACACGGGCACG
cMYC	AAGACAGCGGCAGCCCGAAC	TGGGCGAGCTGCTGTCGTTG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
JUN	GTCCGCACTGATCCGCTCCG	GGGCTGCGCGCACAAGTTTC
MAPK1	TTCCCAGTTCTTGACCCCTG	GTACATACTGCCGCAGGTCA
MAPK3	ACCTACCTAAGGAGCGGCTG	GGCCTCAGCAAAGGAGAGAG
MAPK10	TTGCACTCTGACCATGTTGGTG	TCATCAACCATCCACTTCCTGTCT
NFKB1	GCAGATGGCCCATACCTTCAA	TTGTGAAGCTGCCAGTGCTA
PDGFRA	GCGCAATCTGGACACTGGGA	ATGGGGTACTGCCAGCTCAC
PIK3CA	AACAATGCCTCCACGACCAT	TCACGGTTGCCTACTGGTTC
PLCG1	GCCCGACATCTGCCAAAGAA	AGTCCATTGTCCACCACAAACT
PRKCA	TTTTCCCGGGCAACGACTC	CGCACCCGGACAAGAAAAAG
RELA	GCGAGAGGAGCACAGATACC	GGGGTTGTTGTTGGTCTGGA
TGFB1	GGGCTACCATGCCAACTTCT	GACACAGAGATCCGCAGTCC
WNT3A	CTGCCTGAGGGTGGGCTTTT	TGGAACCTTCCCAGCTCGAC
WNT5A	CTTCGCCCAGGTTGTAATTGAAGC	CTGCCAAAAACAGAGGTGTTATCC
WNT9A	CAGCAGCAAGTTCGTCAAGC	TTGCCCACCTCATGGAAAG
WNT11	TCAGAATGTTCTGCGGGACC	CCGAGTTCACTTGACGAGGC
DUSP3	ATGCACGTCAACACCAATGC	ATGCTCAGGGCAGACTTGAC
POU5F1	AACCTGGAGTTTGTGCCAGGGTTT	TGAACTTCACCTTCCCTCCAACCA
MESP1	TAGGCCTCAGCGAGGAGAGT	TCCCTTGTCACTTGGGCTCC
TBX3	TGGCCTACCATCCGTTCCTA	GGACATCCACTGTTCCCCAG
HXN4	CAGCCTCTTACGCCTGTTAC	CCAGGAGTTGTTCACCATGTTG
SHOX2	CTTACGGCGTTCGTCTCCAA	GACACCTCCGTCAGTCGC
NKX2-5	CGCCGCTCCAGTTCATAG	GGTGGAGCTGGAGAAGACAGA
TBX5	CTCAGTCCCCCGGAACAAC	CACGTACCTCCCAGCTCAAG

are indicated in Table 8. A positive control for phosphorylated Akt (Cell Signaling Technology, Danvers, MA; catalog number 9273S, lot number 20) was diluted 1:2. The anti-rabbit and anti-mouse secondary antibodies included in the Wes Detection Module kit (ProteinSimple, San Jose, CA) were used. All data were analyzed with the Compass Software associated with the Wes instrument (ProteinSimple, San Jose, CA). Data were exported to Prism for further analysis, as described below.

hWnt5a Treatment of CPCs

Neonatal CPCs were grown until approximately 85% confluent and treated with 100ng/mL recombinant human/mouse Wnt5a (R&D Systems, Minneapolis, MN) in CPC growth media for 1 hour, a concentration and duration that were previously shown to induce protein kinase C activity (Koyanagi, et al, 2009). Cells were then washed with PBS and placed in TRIzol® reagent or formed into protein lysates, as described by Abrahamsen, et al. (2013). RT-PCR and western blotting were performed, as described above.

Statistical Analysis

The Shapiro-Wilk test for normality was used to test the normality of data distribution. Student's t-test was used to compare the mean of all normally distributed data. Non-normally distributed data were compared using a Wilcoxon matched-pairs signed rank text. For protein expression analysis, either Student's t-test or Mann-Whitney U test was used to compare the mean of normally or non-normally distributed data, respectively. All data are reported as the mean ± the standard error of the mean. Prism 7

version 7.02 (GraphPad, La Jolla, CA) was used for all statistical analyses. P values < 0.05 were assumed to indicate statistical significance.

Results

Flight aboard the ISS Activates PKCα in Neonatal CPCs

Observations under both simulated microgravity (SMG) and after culture aboard the International Space Station (ISS) prompted us to determine whether calcium handling and signaling were altered by spaceflight. We evaluated the expression of genes involved in regulating intracellular calcium levels and subsequent signaling events in neonatal CPCs after flight aboard the ISS and observed significantly elevated levels of *RYR2* (5.265 ± 1.508-fold change, P<0.05, n=3), *CACNA1S* (8.059 ± 1.771-fold change, P<0.05, n=3), and *CAMK2A* (5.979 ± 1.289-fold change, P<0.05, n=3) (Figure 19A). Changes in calcium handling at 12 days suggested an ultimate induction of calcium signaling at 30 days, which was supported by significantly elevated levels of *PLCG1* (6.041 ± 0.527-fold change, P<0.01, n=3), *PRKCA* (4.482 ± 0.617-fold change, P<0.01, n=3), and *CAMK2A* (3.982 ± 0.540-fold change, P<0.01, n=3) transcripts (Figure 19B). Moreover, we assessed a significant increase in phosphorylated protein kinase C alpha, a calcium-dependent protein kinase C (P-PKCα: 1.448 ± 0.065-fold change, P<0.01; P-PKCα/PKCα: 1.298 ± 0.058-fold change, P<0.05; P-PKCα/GAPDH: 1.466 ± 0.088-fold change, P<0.05; n=3–4) after 30 days of ISS culture (Figure 20A-B). Such ISS-mediated changes to calcium signaling may have a direct impact on cardiogenesis, as shown in the schematic in Figure 20C.

Table 8. Antibodies used for western blot experiments of ISS-cultured and hWnt5a-treated neonatal CPCs

Antibody	Manufacturer	Sample Used	Antibody Dilution	Species	Clone	Catalog No.	Lot No.
GAPDH	Cell Signaling	0.4 mg/mL	1:50	Mouse	D4C6R	97166T	3
AKT (pan)	Cell Signaling	0.04 mg/mL	1:50	Rabbit	C67E7	4691S	17
Phospho-AKT (Ser473)	Cell Signaling	0.4 mg/mL	1:10	Rabbit	D9E	4060S	23
p44/42 MAPK (Erk1/2)	Cell Signaling	0.04 mg/mL	1:25	Rabbit	137F5	4695S	14
Phopsho-p44/42 MAPK (Erk1/2) (Th202/Ty204)	Cell Signaling	0.4 mg/mL	1:10	Rabbit	D13.14.4E	4370S	12
PKCα	Cell Signaling	0.04 mg/mL	1:25	Rabbit	Polyclonal	2056T	4
Phospho-PKCα/β II (Thr638/641)	Cell Signaling	0.04 mg/mL	1:10	Rabbit	Polyclonal	9375T	4

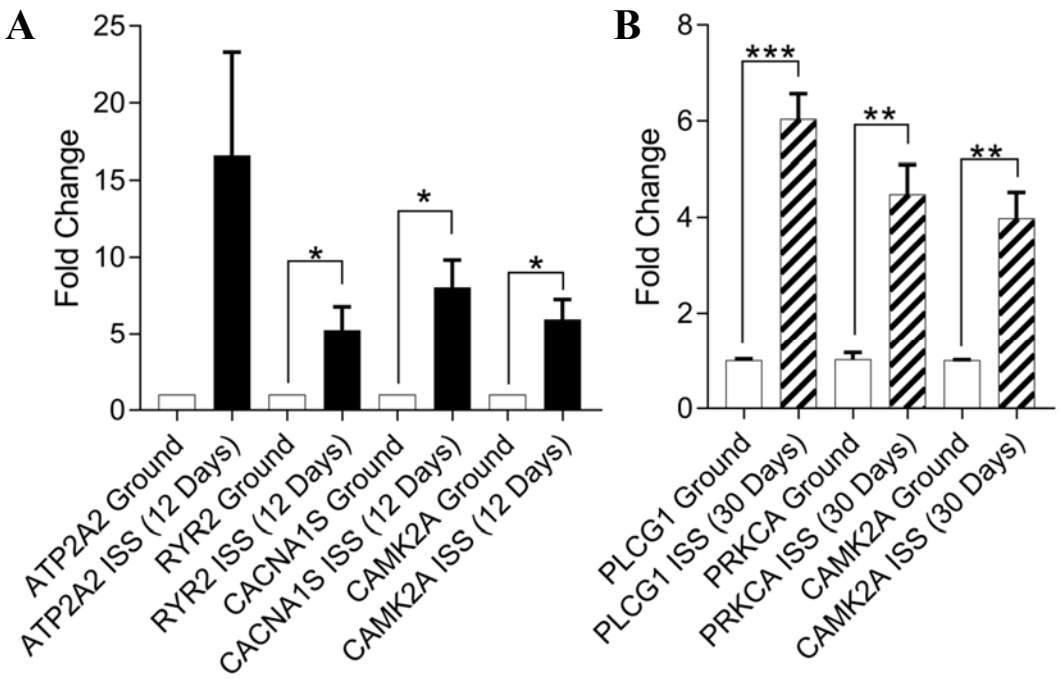


Figure 19. **Spaceflight activates protein kinase C alpha in neonatal CPCs after 12 days** After 12 days aboard the ISS, neonatal CPCs were fixed and induction of genes involved in calcium handling was measured via RT-PCR (A). Calcium handling alterations at 12 days led to the induction of genes involved in calcium signaling at 30 days (B). n=3 per group for RT-PCR; *p<0.05, **p<0.01, ***p<0.001. Fold changes are shown as the mean ± S.E.M.

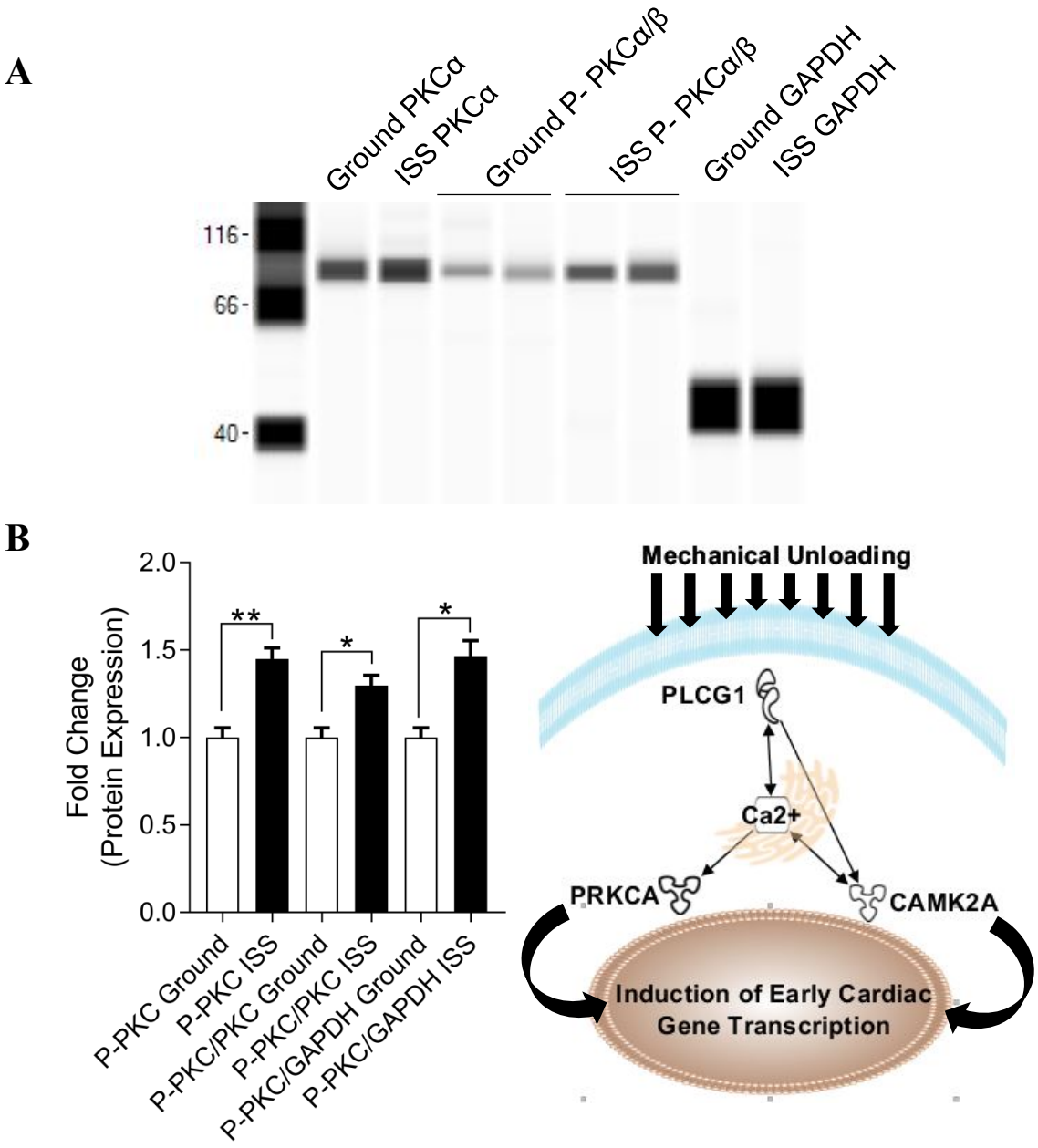


Figure 20. **Spaceflight activates protein kinase C alpha in neonatal CPCs after 30 days** After 30 days aboard the ISS, protein lysates were made from pooled neonatal clones. Phosphorylated PKCαβ, PKCα, and GAPDH protein levels were determined in technical replicates (A-B). ISS-culture mediates changes to calcium signaling that impact cardiogenesis (C) n=3 measurements of four pooled neonatal clones for protein analysis; *p<0.05, **p<0.01, ***p<0.001. Fold changes are shown as the mean ± S.E.M.

Flight aboard the ISS Activates Akt in Neonatal CPCs

These findings of increased expression of calcium-related signaling prompted us to assess the role of calcium-sensitive pathways that are pertinent to stem cell physiology (i.e., ERK and Akt). We assessed the expression of Akt-related signaling genes in neonatal CPCs after 12 and 30 days of flight aboard the ISS. At 12 days, we observed significantly elevated levels of *PIK3CA* (2.216 ± 0.343-fold change, P<0.01, n=9)*, cMYC* (4.811 ± 1.341-fold change, P<0.05, n=9) and *NFκB1* (2.704 ± 0.528-fold change, P<0.05, n=9). At 30 days, we observed significantly elevated levels of *PIK3CA* (7.764 ± 0.472-fold change, P<0.001, n=3)*, CCNDI* (3.257 ± 0.195-fold change, P<0.01, n=3)*, cMYC* (3.297 ± 0.309-fold change, P<0.01, n=9), *JUN* (4.997 ± 0.213-fold change, P<0.001, n=3), *NFκB1* (5.935 ± 0.830-fold change, P<0.01, n=3), and *RELA* (2.799 ± 0.291-fold change, P<0.01, n=3) (Figure 21). Protein expression analysis revealed an induction of phosphorylated Akt following spaceflight when normalized to GAPDH or analyzed as total substrate (P-AKT: 1.573 ± 0.152-fold change, P<0.05; P-AKT/GAPDH: 1.812 ± 0.176-fold change, P<0.05; n=3) after 30 days of ISS culture (Figure 22A-B).

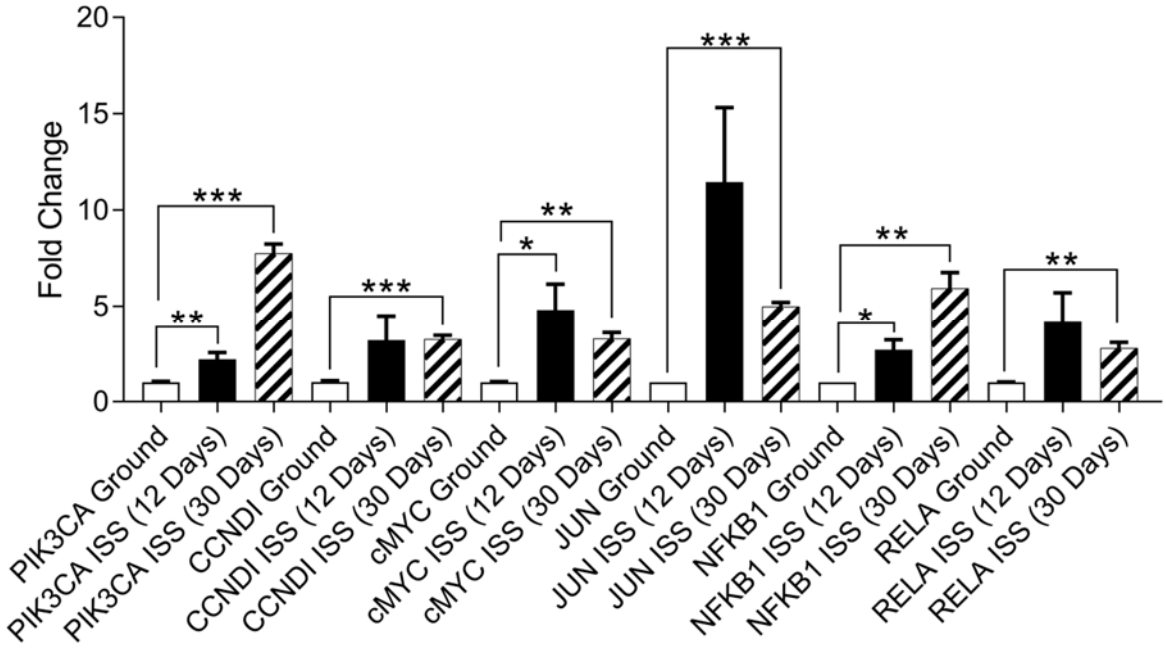


Figure 21. Spaceflight activates Akt signaling in neonatal CPCs
After 12 and 30 days aboard the ISS, neonatal CPCs were fixed and gene expression along the Akt pathway was measured via RT-PCR. n=3–9 per group for RT-PCR; *p<0.05, **p<0.01, ***p<0.001. Fold changes are shown as the mean ± S.E.M.

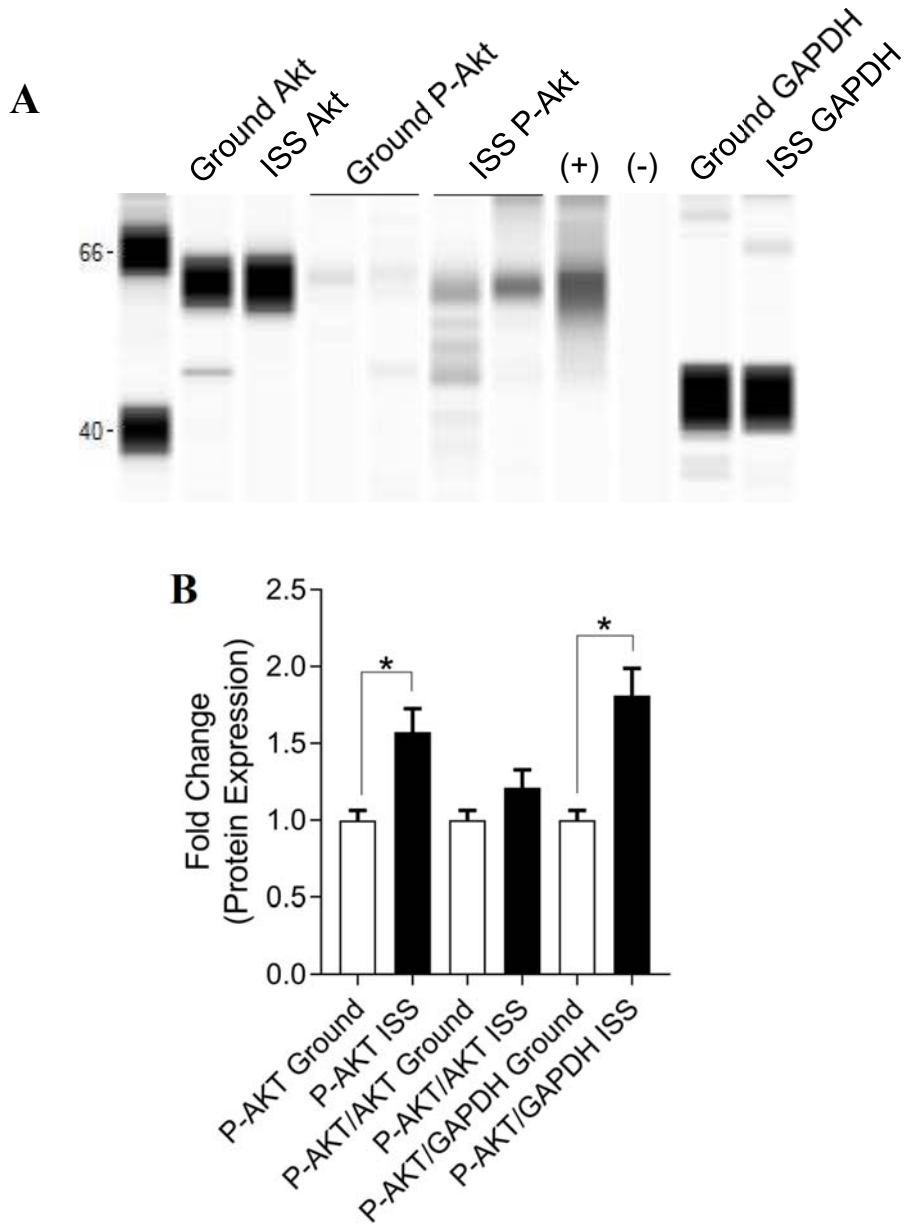


Figure 22. Spaceflight activates Akt signaling in neonatal CPCs
After 30 days aboard the ISS, neonatal CPCs were fixed and protein lysates were made from pooled neonatal clones. Then, phosphorylated Akt, Akt, and GAPDH protein levels were determined in technical replicates (A) and quantified (B). n=3–9 per group for RT-PCR and n=3 measurements of four pooled neonatal clones for protein analysis; *p<0.05, **p<0.01, ***p<0.001. Fold changes are shown as the mean ± S.E.M.

ERK/MAPK is Not Activated in Neonatal CPCs by Spaceflight

This change in Akt activity prompted us to investigate whether ERK/MAPK signaling was altered by spaceflight. We evaluated the expression of ERK/MAPK-related signaling genes in neonatal CPCs after 12 and 30 days of flight aboard the ISS (Figure 23-24). At 12 days, we observed significantly reduced levels of *DUSP3* (0.280 ± 0.126-fold change, $P<0.01$, n=3), which encodes a phosphatase involved in the deactivation of ERK/MAPK signaling. Interestingly, the targets of ERK/MAPK signaling were also elevated: *GRB2* (11.660 ± 2.709-fold change, $P<0.05$, n=3) and *RAF1 (*5.230 ± 0.762-fold change, $P<0.01$, n=3) (Figure 23A-B). However, at 30 days, the induction of genes along this signaling pathway was muted. Moreover, we observed no increase in phosphorylated ERK after 30 days of ISS culture (Figure 24A-B).

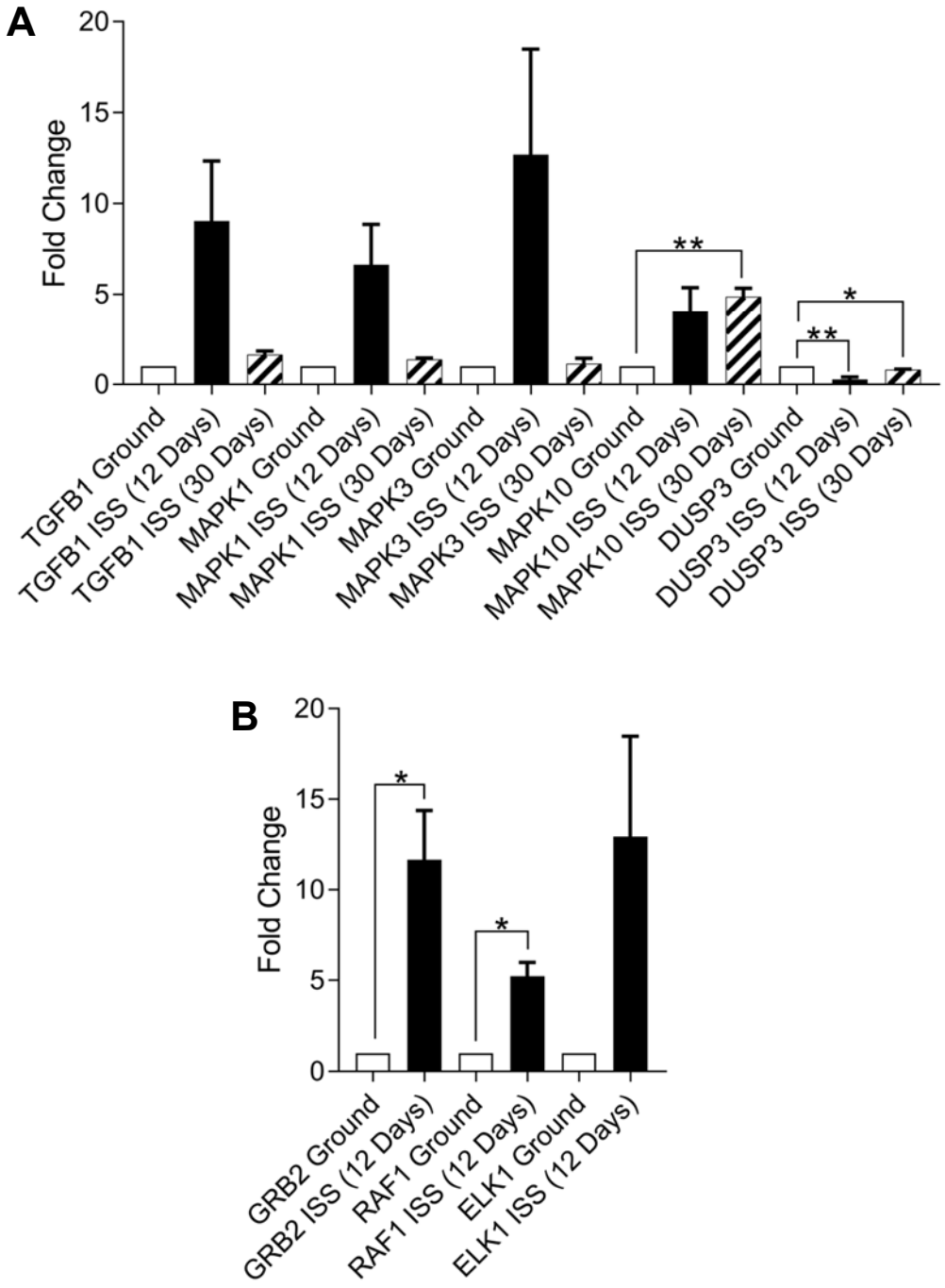


Figure 23. Spaceflight does not activate Erk signaling in neonatal CPCs
After 12 and 30 days aboard the ISS, neonatal CPCs were fixed and gene expression along the ERK/MAPK pathway was measured via RT-PCR. At 12 days, ERK/MAPK pathway (A) and target (B) gene expression was generally elevated; however, at 30 days, such pathway gene expression was largely muted (A). n=3 per group for RT-PCR; *$p<0.05$, **$p<0.01$. Fold changes are shown as the mean ± S.E.M.

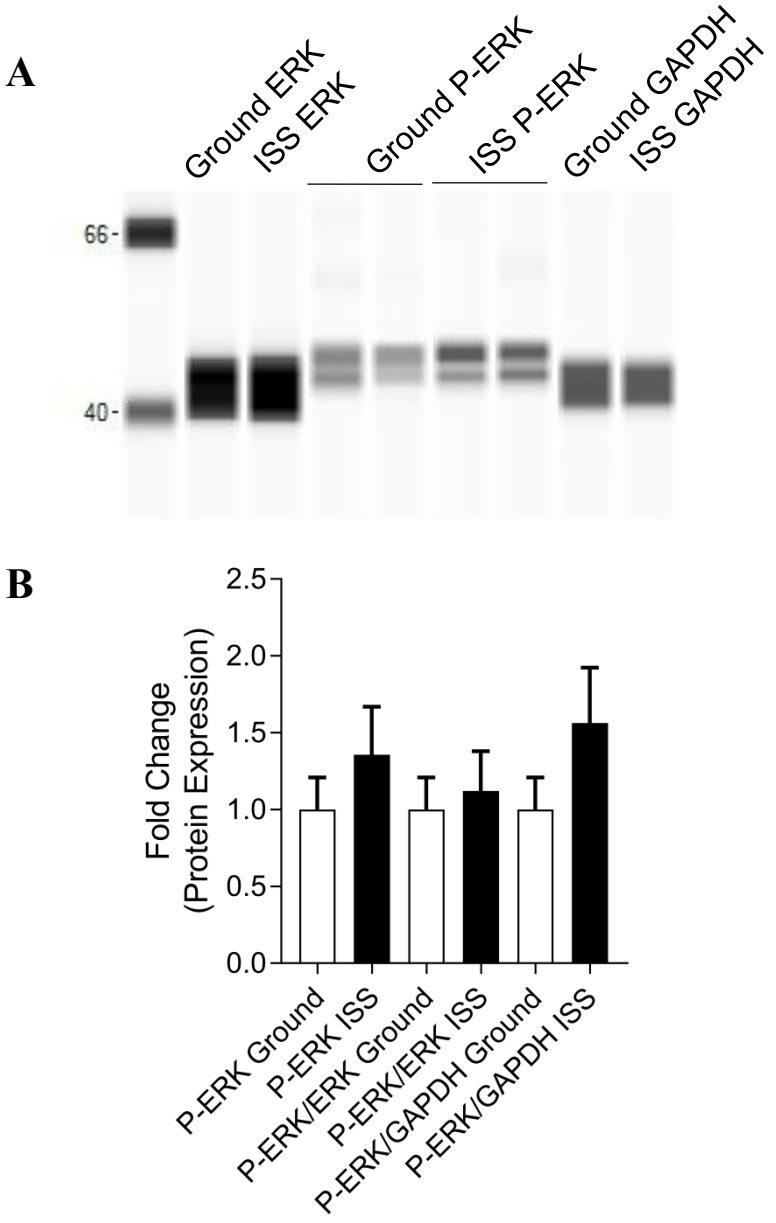
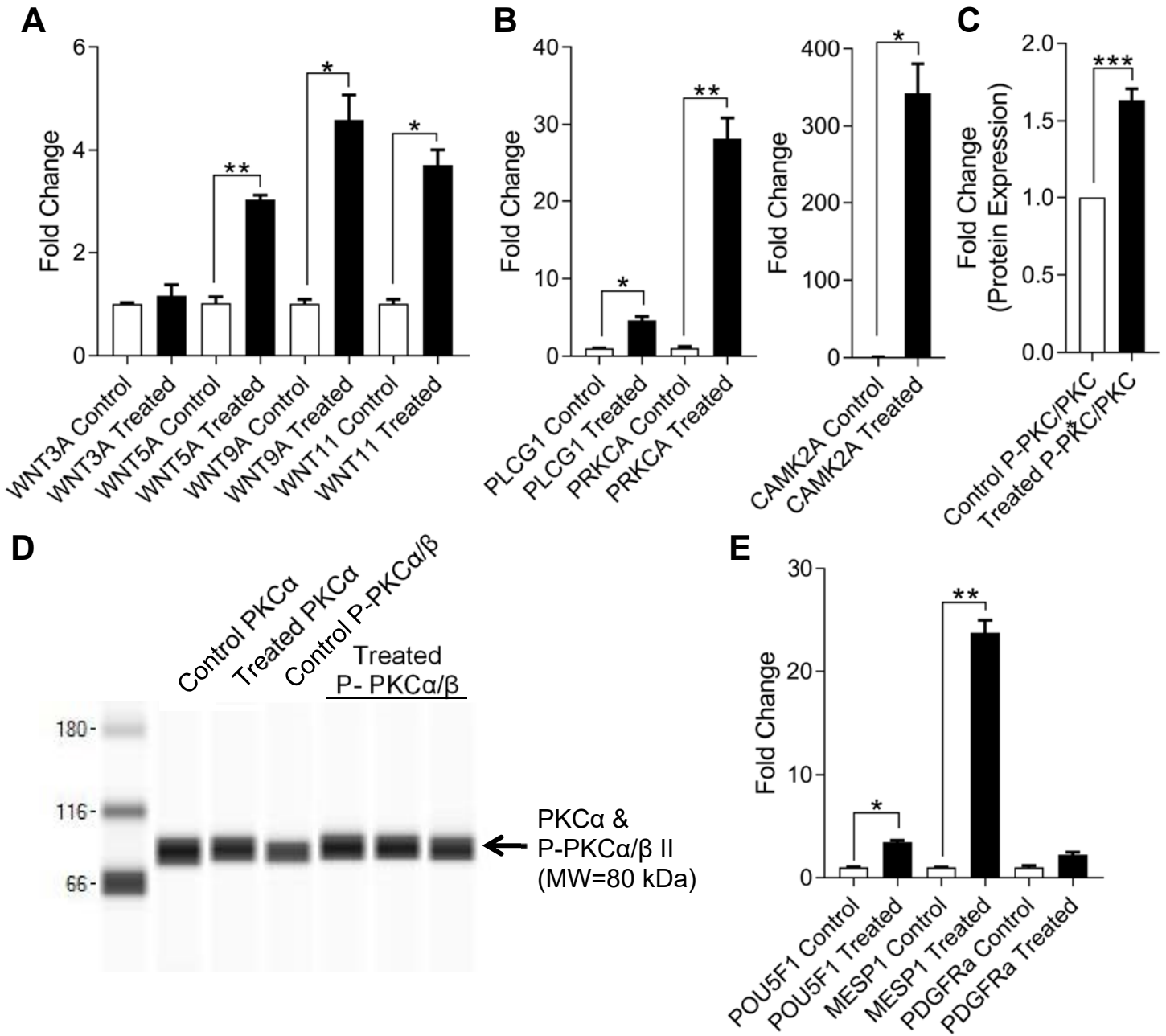


Figure 24. Spaceflight does not activate Erk signaling in neonatal CPCs
Protein lysates were generated from pooled neonatal clones after 30 days aboard the ISS and phosphorylated ERK1/2, ERK1/2, and GAPDH protein levels were determined in technical replicates (C-D). n=3 per group for RT-PCR and n=3–4 measurements of four pooled neonatal clones for protein analysis; *p<0.05, **p<0.01. Fold changes are shown as the mean ± S.E.M.

Ca^{2+} Signaling Contributes to Induction of Genes Involved in Early Cardiogenesis

Given the role of calcium signaling in mediating the effects of spaceflight in neonatal CPCs, we sought to assess the effects of such signaling on the ground. Neonatal CPCs were treated with recombinant hWnt5a (100ng/mL) for 1 hour, which is a concentration and duration that were previously shown to induce PKC activity (Koyanagi, et al, 2009). We then investigated the induction of canonical and non-canonical Wnt ligands (Figure 25A) and calcium signaling genes (Figure 25B): *WNT5A* (3.027 ± 0.087-fold change, P<0.01, n=3), *WNT9A* (4.587 ± 0.485-fold change, P<0.05, n=3), *WNT11* (3.713 ± 0.297-fold change, P<0.05, n=3), *PLCG1* (4.609 ± 0.544-fold change, P<0.05, n=3), *PRKCA* (28.190 ± 2.658-fold change, P<0.01, n=3), and *CAMK2A* (342.700 ± 37.760-fold change, P<0.05, n=3). The induction of PKCα phosphorylation indicated the presence of calcium activity (P-PKC/PKC: 1.635 ± 0.072-fold change, P<0.05, n=3; (Figure 25C-D). This occurred concomitantly with the induction of early developmental genes (Figure 25E), including the pluripotency marker *POU5F1* (3.453 ± 0.194-fold change, P<0.05, n=3) and the mesodermal marker *MESP1* (23.760 ± 1.230-fold change, P<0.01, n=3).


A
Fold Change
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B
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PLCG1 Control
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PRKCA Control
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CAMK2A Control
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C
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Treated P-PKC/PKC
D
Control PKCα
Treated PKCα
Control P-PKCα/β
Treated P- PKCα/β
180-
116-
66-
PKCα &
P-PKCα/β II
(MW=80 kDa)
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Fold Change
POU5F1 Control
POU5F1 Treated
MESP1 Control
MESP1 Treated
PDGFRa Control
PDGFRa Treated

Figure 25. Non-canonical Wnt/Ca^{2+} signaling impacts the developmental phenotype of neonatal CPCs
Wnt5a treatment of neonatal CPCs for 1 hour induces expression of non-canonical Wnt ligands (A), genes involved in the non-canonical Wnt/Ca^{2+} pathway (B), and calcium-dependent PKCα activation (C-D). Induction of genes along this pathway occurred concomitantly with modifications in expression of markers of early cardiogenesis (E). n=3; *$p<0.05$, **$p<0.01$, ***$p<0.001$. Changes in gene expression are shown as the mean ± S.E.M.

Sinoatrial Nodal Genes Are Induced by Calcium Pathway Activity

Increasing evidence indicates that sinoatrial nodal cells represent a population of embryonic myocardium that retains its primitive phenotype (Bakker, 2010). For this reason, we assessed the relationship between primordial cardiogenesis gene induction and that of the sinoatrial nodal gene program in Wnt5-treated CPCs (Figure 26). We observed significant increases in the expression of *TBX3* (5.212 ± 0.917-fold change, $P<0.05$, n=3), *TBX5* (7.678 ± 0.178-fold change, $P<0.01$, n=3), *SHOX2* (6.318 ± 0.813-fold change, $P<0.05$, n=3), and *HCN4* (9.489 ± 1.191-fold change, $P<0.05$, n=3) along with decreased expression of *NKX2-5* (0.237 ± 0.044-fold change, $P<0.05$, n=3).

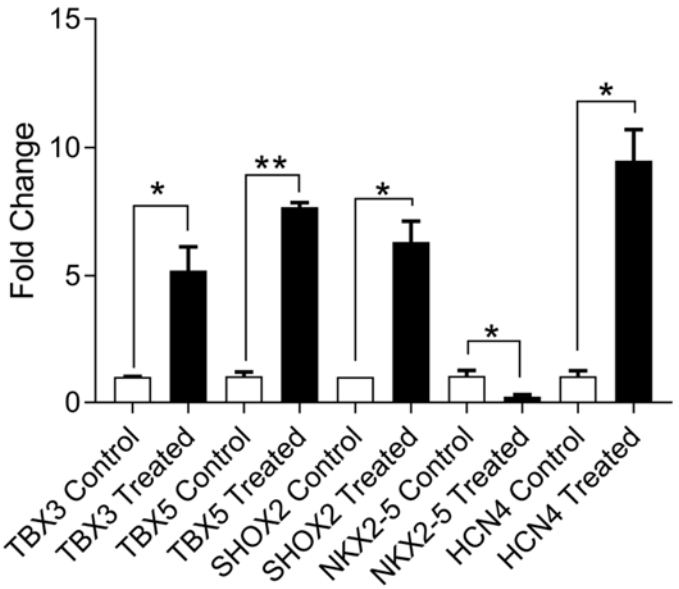

Figure 26. SAN gene induction via calcium signaling activation
Wnt5a treatment of neonatal CPCs induces sinoatrial nodal gene and protein expression. The primordial phenotype of Wnt5a-treated CPCs included the induction of sinoatrial nodal genes. n=3; *p<0.05, **p<0.01, ***p<0.001. Changes in gene expression are shown as the mean ± S.E.M.

Discussion

Culture aboard the ISS resulted in the activation of calcium-dependent protein kinase C (PKCα) and Akt in neonatal CPCs. This increase in calcium signaling was modeled *in vitro* on Earth using hWnt5a, a non-canonical ligand that promotes protein kinase c activation. We observed an important role in calcium signaling in accounting for the effects, at least in part, of spaceflight within neonatal CPCs.

Among the changes experienced by CPCs during spaceflight, we found that calcium pathway genes and those related to calcium handling were expressed at higher levels following culture aboard the ISS as well as under SMG, though to a more modest extent. These changes in calcium extend beyond human CPCs. During spaceflight, calcium loss is widely observed in astronauts and is usually linked to changes in bone metabolism (Smith, 2012). Yet, in one animal model of SMG, mice were observed to exhibit an increased incidence of arrhythmias along with alterations in intracellular calcium handling, including ryanodine receptor (RyR2) phosphorylation (Respress, 2014). Moreover, short-duration atrial fibrillation, premature ventricular contractions, and ventricular tachycardia have all been reported in astronauts during spaceflight (Anzi, 2014). Importantly, while the etiology of such cardiac events during spaceflight remains unknown, disturbances in calcium handling may be an important contribution to cardiac abnormalities in space.

Meanwhile, the modification of signaling pathways related to calcium, either as an important secondary or mediating molecule, directly impacts the ability of CPCs to differentiate or maintain pluripotency (Tonelli, 2012; Apati, 2016). The effect of calcium signaling is dependent both on the pathway in which it is involved as well as the species

under study, with mouse ESCs exhibiting far different responses to calcium-mediated activity compared to human ESCs. For example, calcium signaling promotes pluripotency in hESCs, while being affiliated with differentiation in mESCs (Tonelli, 2012). In the context of cardiogenesis, studies performed in both embryos and ESC-derived cardiomyocytes have shown a critical role of Ca^{2+} in regulating multiple steps of heart formation (Pucéat, 2005). For example, induction of Ca^{2+} oscillation promotes proliferation and, upon transplantation, enhances engraftment and expansion (Ferreira-Martins, 2009). In this way, our observed shift in expression of markers of early cardiac development is likely the result, at least in part, of modified calcium signaling activity within neonatal CPCs. Furthermore, research into Mesp1-expressing pre-cardiac mesoderm derived from human ESCs exhibited enriched activity along the calcium, extracellular matrix-receptor, and Wnt signaling pathways at day 5 of development (den Hartogh, 2016), which is supported by our observed increase in markers of such a state of development after culture aboard the ISS. Meanwhile, Akt signaling via calcium activation has been well documented to promote the maintenance of pluripotency in hESCs (Tonelli, 2012). Additionally, Akt signaling exerts a critical role in several cell functions that are relevant to stem cell transplantation, migration, and cytokine expression (Manning, 2007). Therefore, manipulating calcium signaling on Earth and promoting Akt activation presents a novel therapeutic opportunity for cell-based cardiac repair.

In addition to modifying intracellular calcium signaling, the expression of non-canonical Wnt ligands has been observed in association with the cryoinjury response of the neonatal rodent heart (Mizutani, 2016). Since *WNT5A* and *PRKCA* gene expression

induction is involved in the response of neonatal CPCs to SMG, we sought to assess the potential role of Wnt5a in promoting enhanced stemness in CPCs. While preliminary, these early results indicate a relationship between enhanced intracellular calcium signaling as well as increased expression of markers of early cardiogenesis in neonatal CPCs. The expression of early cardiogenic mesoderm markers indicates a potentially earlier developmental state. In addition to being linked to enhanced therapeutic potential following transplantation (Menasché, 2015; Behfar, 2010; Bartunek, 2013; Blin, 2010), such an early developmental state may also provide the appropriate cell source for biological pacemaker development. In an avian model, Bressan et al. used fate mapping to determine that sinoatrial nodal cells were observed to already be specified shortly after gastrulation prior to the onset of cardiogenesis within a region that was observed to be Nkx2-5- and Isl1-negative (Bressan, 2013). Meanwhile, the Keller group recently reported a method of generating sinoatrial nodal-like pacemaker cells in Nkx2-5-negative cardiomyocytes (Protze, 2017). Similarly, we observed decreased *NKX2-5* and *ISL1* expression under both SMG and MG. When considered together, our findings using simulated and real MG support the emerging hypothesis that sinoatrial nodal cells represent a de-/un-differentiated state of cardiac development (Bakker, 2010). Moreover, our observation that calcium signaling has an integral role in this process is mirrored by observations that Wnt7a, which mobilizes intracellular calcium (Thrasivoulou, 2013), has been observed in the developing cardiac conduction system (Gessert, 2010). Given the observed changes to the expression of calcium handling genes and proteins, future studies should assess the effect of SMG and ISS-culture on intracellular Ca^{2+} transients and the electrophysiological properties of CPCs.

Notably, the increased expression of sinoatrial nodal markers may reflect the emerging hypothesis that this unique functional cell type develops from myocardium that retains its primitive phenotype (Bakker, 2010). Accordingly, the concomitant increased expression of sinoatrial nodal genes and decreased expression of other mesodermal derivatives indicates that the reduced gravity conditions of spaceflight promote the modest de-differentiation of neonatal CPCs. In our previous chapter, we documented an significant decrease in the expression of microRNA-106b, which otherwise would repress the induction of the sinoatrial nodal gene program. Additionally, the elevated expression of Tbx proteins, particularly, Tbx3, overlaps with previous reports in the regulation of pre-mesoderm (i.e., mesendoderm) gene expression (Weidgang, 2013). As described in Baio et al. (2018), a similar induction of the sinoatrial nodal gene program was observed in SMG-treated CPCs: *TBX3* (141.600 ± 54.550-fold change, $P<0.05$, n=3), *TBX5* (35.620 ± 4.667-fold change, $P<0.05$, n=3), *HCN4* (48.510 ± 18.710-fold change, $P<0.05$, n=3), and *NKX2-5* (0.146 ± 0.042-fold change, $P<0.001$, n=3).

Importantly, many of these processes observed reduced gravity conditions are dependent upon the developmental status of the cell. Indeed, later stages of cardiogenesis and the incipient developmental cues of embryonic stem cells all respond differently to calcium and Wnt signaling (Ueno, 2007; Cohen, 2008; Ozhan, 2015). Therefore, understanding the effects of mechanical transduction and altered intracellular calcium signaling within the myriad cell types that constitute the human body will help inform medical interventions that will be necessary to sustain deep space missions. Meanwhile, the molecular events that constitute microgravity sensing can be manipulated on Earth to

facilitate regeneration. In doing so, microgravity-inspired, cell-based therapies can be developed.

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CHAPTER FOUR

ANGIOTENSIN II TREAMENT OF NEONATAL CARDIOVACULAR PROGENITOR CELLS INDUCES THE DEVELOPMENT OF SINOATRIAL NODAL-LIKE CELLS

Abstract

The cardiac pacemaker has been used to treat arrhythmias that arise from dysfunction of the sinoatrial node (SAN), which generates the pacing activity of the heart. Though these pacemakers are limited in responsiveness to autonomic regulation and require routine maintenance, no alternative therapies exist. Primordial Islet-1+ cardiovascular progenitor cells (CPCs) differentiate into functional pacemaker cells during development. Transcription factors from the T-box family, including Tbx3, Tbx5, and Tbx18, are implicated in this process. We have previously shown that simulated microgravity, International Space Station culture, and hWnt5a treatment induced activity along the phospholipase C (PLC)/protein kinase C alpha (PKCα)/calcium signaling axis and that these conditions correlated with the induction of sinoatrial nodal-related gene expression. Therefore, we induced PLC/PKCα/Ca^{2+} signaling via angiotensin II receptor type I activation to differentiate Islet-1+ human neonatal-derived CPCs into functional SAN cells that express the characteristic funny (i.e., pacemaker) current-carrying hyperpolarization-activated cyclic nucleotide gated (Hcn4) channel. Following treatment with angiotensin II for 72 hours, RT-PCR was used to assess expression of genes of the SAN gene program, which was found to be induced. This resulted in a 7.75-fold increase in Hcn4 protein induction, as indicated by western blotting. Furthermore, cardiomyocyte

markers, including transcripts of TropT and Cxn43, were all significantly decreased in expression. Treated CPCs were then stained with fluo4-AM, an intracellular calcium dye, imaged using confocal microscopy, and observed for the presence of an auto-rhythmic calcium flux. Electrophysiology recordings of treated cells in the whole cell, perforated patch configuration revealed an action potential of -40 ± 1 mV, a slow maximum upstroke velocity of $dV/dt_{max} < 10$ V/s, and an epinephrine sensitivity that accelerated the number of action potentials from 1.05 ± 0.05 to 1.41 ± 0.05 per second.

Introduction

Atrial arrhythmias represent a group of common medical conditions that annually affect more than three million individuals in the United States (NHLBI, 2014). While many of these patients can address these fibrillations through changes in lifestyle, expanded access to resources related to health improvement, or with medication, over 180,000 annual patients still require an exogenous pacemaker (Greenspon, 2012). Many complications can result from both implanting and using a pacemaker, including infections during implantation, parenchyma erosion, hemo- and pneumothorax, pacemaker migration or dislodgement, and venous thrombosis (Gul, 2011). Lead displacement occurs in 5–10% of pacemaker recipients (National Pacemaker and ICD database, 2001), while pacemaker syndrome (i.e., atrioventricular asynchrony) and other long term complications of pacemakers are estimated to occur in approximately 2.7% of patients (Trohman, 2004). For children who are born with a congenital arrhythmia, pacemaker implantation must be performed regularly to keep pace with the developing size of the child (Cho, 2015). Among all patients, magnetic interference, such as from portable headphones (Lee, 2009) and magnetic resonance imaging scanners (Kalin, 2005), are clinical concerns due to their ability to induce asynchronous pacing. For this reason, a biological pacemaker, such as a CPC-derived sinoatrial node, has been the subject of recent research efforts.

Application of Reduced Gravity Conditions on Earth

Lessons for sinoatrial nodal development may be informed by studies of cardiovascular precursors under conditions of microgravity. Simulated microgravity has

been shown in our lab and elsewhere to result in the expansion of pacemaker cells or of markers of the sinoatrial nodal gene program (Hatzistergos et al., 2018; Baio, 2018). We also demonstrated in Baio et al. (2018) that *TGFB1* expression was induced under these conditions, which supports recent work by the laboratory of Ann C. Foley, who demonstrated that MAP3K7, or TGFß-activated kinase 1, overexpression in mouse embryonic stem cells faithfully induced sinoatrial node development (Brown, 2017).

Indirectly, the reduction in NKX2-5 expression in cardiovascular progenitors during spaceflight indicates that reduced gravity conditions may prime the cell population under study to assume a sinoatrial nodal gene expression profile. As shown in Bressan et al. (2013) and Protze et al. (2017), pacemaker cells are specified shortly after gastrulation and prior to the onset of cardiogenesis in a population of Nkx2-5-negative cells. This is mirrored by a similar reduction in Nkx2.5 expression in SMG-treated CPCs, whose sensitivity to gravity is modulated, at least in part, by calcium signaling (Baio, 2018). Interestingly, calcium signaling has been shown to be activated in the developing cardiac conduction system via Wnt7a induction (Gessert, 2010), which mobilizes intracellular calcium (Thrasivoulou, 2013).

In particular, the association between sinoatrial nodal gene program induction and calcium signaling under simulated microgravity (Baio, 2018), during culture aboard the International Space Station, and following treatment with hWnt5a, indicates that calcium potentiation may be one method of creating an enriched sinoatrial nodal cell population on Earth. Various pathways can achieve this end, including non-canonical Wnt signaling (e.g., Wnt5a) and angiotensin II receptor type I activation (Figure 27). While both processes may be applied, the latter has been carefully studied in the cardiac context. In

rodent models, angiotensin II receptor activation increases ERK phosphorylation, nuclear [Ca^{++}] levels, and proliferation (Tadevosyan, 2012). Moreover, angiotensin II receptor type I activation and antagonism has been shown to modulate hyperpolarization activated, cyclic-nucleotide gated potassium/sodium channel 4 (Hcn4) and the sinoatrial nodal-characteristic funny current (Xu, 2009). Finally, pacemaker-like cells have been shown to directly be induced from mouse cardiac progenitor cells via angiotensin II treatment (Xue, 2015). Thus, we sought to test whether the sinoatrial nodal-like phenotype that emerges following exposure of progenitor cells under reduced gravity conditions is mediated, at least in part, by calcium signaling. The molecular biology of sinoatrial nodal development has been discussed earlier (chapter one), while the functional properties governing sinoatrial nodal cell function are described below. Knowledge of these properties is foundational to verifying the authenticity of sinoatrial nodal cell induction.

Functional Properties of the Sinoatrial Node

Beginning in the 1800s, the individual efforts of Jan E Purkinje, Wilhelm His, and Sunao Tawara collectively initiated the elucidation of the conduction system of the heart (Silverman, 2007). Then, in 1906, Martin Flack and Arthur Keith identified a structure that was located at the junction of the superior vena cava and the right atrium of the heart (Keith, 1907). Given its resemblance to the then-recently identified atrioventricular node (Tawara, 1906), Flack and Keith identified this as the sino-auricular node (Silverman, 2007). Subsequent electrophysiology studies confirmed the postulated function of this node as well as resulted in the use of its present name, the sinoatrial node (Lewis).

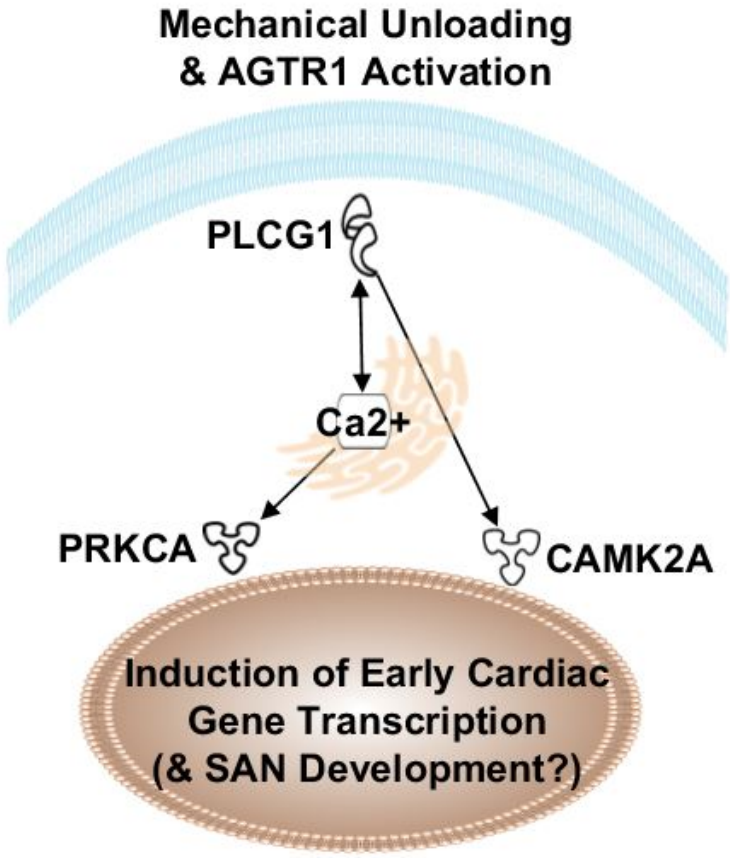


Figure 27. Angiotensin II receptor type I activation induces calcium signaling (Baio, 2018)
As in simulated microgravity, angiotensin II receptor type I activation activates phospholipase C (PLCG1), potentiates calcium, and activates protein kinase C alpha (PRKC). Ultimately, broad cellular activities are modified, including migration, proliferation, and development.

The sinoatrial node initiates the cardiac conduction system in an automated fashion. While other nodes, such as the atrioventricular node, have their own pacing potential, they are suppressed by the higher frequency (60–100 bpm) of the sinoatrial node. The higher frequency of sinoatrial node firing prevents downstream nodes from reaching their pacing potential threshold (Vassalle, 1977). For this reason, the sinoatrial node is often considered the *pacemaker* of the heart.

This automaticity is derived from a series of inward- and outward-directed ion channels (Verkerk, 2007). As shown in Figure 28, following the conclusion of an action potential, there is a slow, positive increase in voltage across the sinoatrial nodal cell membrane (i.e., the pacing potential). When the pacing potential reaches the action potential threshold, a new action potential is fired (Berne, 2004). This slow, positive increase is an important feature of the sinoatrial node. Unlike the resting membrane potential of cardiomyocytes, which is generally stable, the sinoatrial node's resting membrane potential is constantly and slowly increasing from an average potential of -70mV to the average threshold of -40mV (Sherwood, 2012). It is also here where the autonomic nervous system can modulate the rhythm of the heart by accelerating (sympathetic) or decelerating (parasympathetic) the increase in the resting membrane potential (Brown, 1979; Bucchi, 2007; Di Francesco, 1991, 2010).

A variety of ion channels produce the electrical conduction activity of the sinoatrial node (Cho, 2015; Verkerk, 2007), while the regulation of these ion channels, such as by secondary signaling molecules, modulates its automaticity (Sah, 2014; Bucci, 2007). During the slow pacing phase of the heart cycle, or diastolic depolarization in which blood is filling the chambers prior to sinoatrial node action potential firing and signal

propagation, inward ion currents are activated while outward ion currents are deactivated (Verkerk, 2007). Among the currents that are produced by the activated channels of diastolic depolarization, the hyperpolarization-activated pacemaker current, or the funny current (I_f), is considered to be the principal component of the pacemaker and accounts for the autonomic sensitivity of the sinoatrial node and is often implicated in age-related heart conditions (Di Francesco, 1993, 2007; van Ginneken, 1991; Accili, 1997, Verkerk, 2003; Zicha, 2005). The hyperpolarization activated cyclic nucleotide gated potassium channel 4 (Hcn4) is a molecular constituent of the pacemaker current-carrying channel (Stieber, 2004), thereby contributing to its mixed potassium and sodium constitution. In response to hyperpolarization (between -70 mV to -40 mV), the pacemaker current is activated. Furthermore, the secondary messenger, cyclic adenosine monophosphate (cAMP), regulates diastolic depolarization. In response to sympathetic activation, cAMP levels increase, thereby increasing the rate of pacemaker current activation and accelerating the pace of action potential firing in the sinoatrial node (Di Francesco, 1991, 2007). Additional currents that are produced by channel activation during diastolic depolarization include the calcium-release activated sodium-calcium exchange current (I_{NCX}), the sustained inward current (I_{st}), the sodium current (I_{Na}), and the T- and L-type calcium currents ($I_{Ca,T}$ and $I_{ca,L}$) (Verkerk, 2007). Despite the work of Di Francesco and his colleagues in characterizing the role of the pacemaker (i.e., funny) current in pacing, there is controversy over the significance of this current. Other investigators have argued that I_{Na} and $I_{ca,L}$ are the principle currents of the sinoatrial node (Boyett, 2000). Both arguments are supported by substantial evidence, suggesting that these currents behave synergistically, and perhaps in a location-specific manner, to contribute to the slight

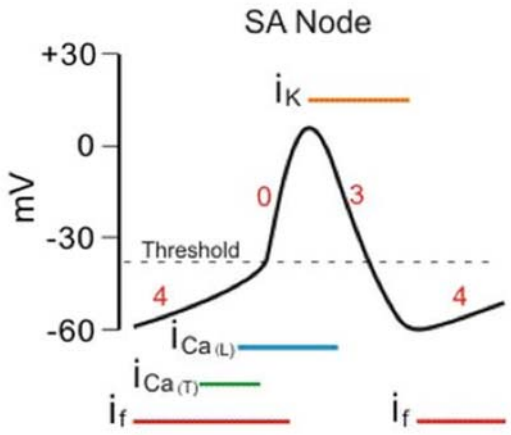


Figure 28. SAN pacing currents (Klabunde, 2011)
Ionic currents drive the gradual depolarization that is characteristic of the SAN. I_f: funny current; $i_{Ca(T)}$: T-type calcium current; $i_{Ca(L)}$: L-type calcium current; i_K: potassium current.

increase in resting membrane potential that accounts for the automaticity of the sinoatrial node.

Interestingly, it has been reported that a radial distribution of cell morphologies exist in the sinoatrial node (Boyett, 2007). The most archetypal pacemaker cells exist in the center, or leading pacemaking site, of the SAN. These cells are smaller, poorly organized, and without many mitochondria. More peripherally, there are atrial-like cells that are still involved in automated electrical conduction but interdigitate and function synergistically with the surrounding atrial tissue. These peripheral cells are observed to be larger and express connexin channel 40 (Cx40) as well as the sodium transporter, Nav1.5. This radially organized and heterogeneous fine architecture of the SAN facilitates its function as peripheral channels may be more resilient to hyperpolarization currents that are derived from atrial tissue, thereby guaranteeing the unidirectional propagation of the pacing potential. Histologically, an abundance of collagen and fibroblasts and an underlying layer of atrial muscle have been observed, perhaps conferring protection to the SAN against atrial wall stress (Boyett, 2000; Dhein, 2009).

Upon reaching the threshold potential for action potential initiation, the sinoatrial node propagates the signal through connexin channel 45 (Cx45; Lo, 2000; Dhein, 2010) into the adjacent atrial cells, thereby inducing atrial contraction and moving blood from the atrium into the ventricles. The gradual intercellular coupling that is afforded by the above-described spatial distribution allows the propagation of the action potential into the atria (Boyett, 2000). This signal eventually arrives at the atrioventricular node, which then descends, following a brief delay that allows the ventricles to fill with blood, down the ventricles through the bundle of His to be terminally distributed through the Purkinje

fibers as well as through the right and left bundle fibers. This final distribution of electrical activity results in ventricular contraction by cardiomyocyte activity, which moves blood from the heart to either the pulmonic or systemic circulation. These cardiomyocytes rapidly depolarize following a rapid influx of sodium ions that is occurs after rapid sodium channels open under the influence electrical activity that is transported from neighboring cells through connexins. These sodium channels are then inactivated while potassium and chlorine channels transiently open, allowing for a small decrease in membrane potential that is characteristic of cardiomyocytes. The membrane potential then plateaus due to a balance between an inward calcium current and an outward potassium current. (Sherwood, 2012). Finally, cardiomyocytes experience a rapid repolarization following the closure of the calcium current producing channels. While cardiomyocytes are generally under the influence of the sinoatrial node, cardiomyocytes can independently contract (Satin, 2004). Indeed, embryonic stem cell-derived cardiomyocytes have already been shown to be able to develop self-generating rhythms of contraction (Kehat, 2004).

The heart is susceptible to atypical pacing following exposure to adverse conditions. For example, following hypoxia, persistent sodium current is observed to increase after cardiomyocyte depolarization (Ju, 1996). Additionally, Weisfeldt et al. (1974) demonstrated the loss of a healthy cardiac conduction cycle that was characterized by a loss of complete relaxation following an action potential. Meanwhile, under conditions of microgravity, such as during spaceflight, heart rate appears to be altered or become variable, possibly due to changes in susceptibility to autonomic influence (Migeotte, 2003; Xiao, 2004). Therefore, studies of pacing in CPC-derived sinoatrial

nodal cells can intuitively be extended to include studies of automaticity in cardiomyocytes following adverse conditions.

Therefore, we sought to develop cells that could ultimately form the basis for biological pacemaker development *in vitro*. To achieve this end, we applied the mechanisms observed in CPCs during spaceflight on Earth. We then screened the induction of the sinoatrial nodal gene program and of *Hcn4* protein. We concluded our experiments with an assessment of sinoatrial nodal physiology using confocal microscopy imaging of calcium flux and electrophysiology studies.

Materials and Methods

Ethics Statement/Cell Isolation and Expansion

CPCs were isolated from cardiac tissue of neonates (1 day – 1 month), as previously described (Fuentes, 2013). The Institutional Review Board of Loma Linda University approved the protocol for use of tissue that was discarded during cardiovascular surgery, without identifiable private information, for this study with a waiver of informed consent. Briefly, atrial tissue was cut into small clumps (approximately 1.0 mm^3) then enzymatically digested using collagenase (Roche, Indianapolis, IN) at a working concentration of 1.0 mg/mL. The resulting solution was then passed through a 40-μm cell strainer. Cells were cloned in a 96-well plate by limiting dilution to a final concentration of 0.8 cells per well to create populations for expansion. Cells were then screened for the co-expression of Isl1 and c-Kit. Clonal CPC cultures were selected for other markers of early cardiac development (KDR and PDGFRA) and then supplemented with growth media comprised of 10% fetal bovine

serum (Thermo Scientific, Waltham, MA), 100 µg/mL penicillin-streptomycin (Life Technologies, Carlsbad, CA), 1.0% minimum essential medium non-essential amino acids solution (Life Technologies, Carlsbad, CA), and 22% endothelial cell growth media (Lonza, Basel, Switzerland) in Medium 199 (Life Technologies, Carlsbad, CA). Mycoplasma contamination was tested using the MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland) following the manufacturer's protocol.

Angiotensin II Dilution and Dosage Titration

Angiotensin II (Sigma Aldrich, St. Louis, MO) was dissolved in cell culture grade water and added to CPC growth media at concentrations of 1, 5, and 10 µM for 48, 72, and 168 hours. The induction of *TBX3* and *HCN4* expression was measured via RT-PCR and flow cytometry at all concentrations to determine that 10 µM of angiotensin II for 72 hours was the optimal treatment condition.

RNA Purification and cDNA Synthesis

Neonatal CPCs that were treated with angiotensin II were fixed in RNA Protect (Qiagen, Valencia, CA). RNA was purified from the RNA Protect samples using the RNeasy Mini Kit (Qiagen, Valencia, CA), per the manufacturer's instructions. cDNA was prepared using 2 µg of RNA with Superscript III (Life Technologies, Carlsbad, CA) and used for gene expression analysis in quantitative RT-PCR experiments, as described below.

Quantitative RT-PCR

Quantitative real-time polymerase chain reaction was performed using Go-Taq® qPCR Mastermix (Promega, Madison, WI) and the iCycler iQ™5 PCR Thermal Cycler (Bio-Rad, Hercules, CA) following a protocol of 94°C for 10 minutes followed by 45 cycles of 94°C for 15 seconds, 52–68°C (depending on the primer) for 60 seconds, and 72°C for 30 seconds. RT-PCR products were visualized using 1–2% agarose gel electrophoresis and low mass DNA ladder (Invitrogen, Carlsbad, CA). Threshold cycle values were then analyzed for each individual clone using the comparative C_T method (Schmittgen, 2008).

Primers were designed using the National Center for Biotechnology Information Primer-BLAST program and obtained from Integrated DNA Technologies (Coralville, IA). Primers used in experiments are listed in Table 9.

Flow Cytometry

We used flow cytometry to measure the expression of Hcn4. Cells were labeled to detect Hcn4 (ab85023; Abcam, Cambridge, MA; clone: S114-10). Isotype controls were matched to the species, isotype, and conjugated fluorophore. Fluorescently labeled cells were analyzed using MACSquant analyzer (Miltenyi Biotec, Auburn, CA) and FlowJo software (FlowJo, Ashland, OR). Dead cells were gated out using forward-scatter and side-scatter gating.

Table 9. Primer pairs used in RT-PCR for SAN experiments (5' to 3')

Gene	Forward Sequence	Reverse Sequence
ACTIN	TTTGAATGATGAGCCTTCGTCCCC	GTCTCAAGTCAGTGTACAGGTAAGC
AGTR1	TGCCTCCTCGCCAATGATTC	TGAAACTGACGCTGGCTGAA
AGTR2	GTGTTTAGGCACTAAGCAAGCTG	GCTAGTAGTGGCAAGGGTGG
CAMK2A	GTCCAGTTCCAGCGTTCAGTT	GTGGGGATTCAGGATGGTGG
GJA1	CATTAGGGGGAAGGCGTGAG	AGTCAGTCAAATCCCCCAACC
GJC1	GAGGTGGAGGAGAGGCGAG	CCGAGCTGCCTTCTTGTCTG
HCN4	CAGCCTCTTACGCCTGTTAC	CCAGGAGTTGTTCACCATGTTG
NKX2-5	CGCCGCTCCAGTTCATAG	GGTGGAGCTGGAGAAGACAGA
PLCG1	GCCCGACATCTGCCAAAGAA	AGTCCATTGTCCACCACAAACT
PRKCA	TTTTCCCGGGCAACGACTC	CGCACCCGGACAAGAAAAAG
SHOX2	CTTACGGCGTTCGTCTCCAA	GACACCTCCGTCAGTCGC
TBX18	GGTGGCAGGTAATGCTGACT	ACTTGCATTGCCTTGCTTGG
TBX3	TGGCCTACCATCCGTTCCTA	GGACATCCACTGTTCCCCAG
TBX5	CTCAGTCCCCCGGAACAAC	CACGTACCTCCCAGCTCAAG
TNNT2	GTGGGAAGAGGCAGACTGAG	ATAGATGCTCTGCCACAGC

Protein Simple

Following treatment with angiotensin II and detachment, CPCs were homogenized using RIPA buffer containing phosphatase inhibitor cocktail (Millipore, Temecula, CA), followed by centrifugation at 14,000 g for 15 minutes at 4°C and collection of the supernatant for analysis. Total protein concentrations were determined using the Pierce Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). A capillary-based western blotting system (ProteinSimple Wes, San Jose, CA) was used to assess protein expression. All procedures were completed according to the manufacturer's instructions and default settings. Mouse, anti-Hcn4 antibody (1:10; cat no. ab85023; Abcam, Cambridge, MA; clone: S114-10) and mouse IgG2b, anti-human β-Actin (1:50; cat no. 3700; Cell Signaling Technology, Danvers, MA; clone: 8H10D10) were used with the anti-mouse detection module from ProteimSimple. All data were analyzed with the Compass Software associated with the Wes instrument (ProteinSimple, San Jose, CA). Data were exported to Prism for further analysis, as described below.

Calcium Handling Analysis

Fifty micrograms of Fluo4-AM dye (Life Technologies, Carlsbad, CA) was dissolved in pluronic acid and 20% DMSO (Life Technologies) via sonication before being resuspended in M199-supplemented growth media. Angiotensin II-treated CPCs were then incubated with the dye for 1 hour and imaged using a LSM 710 NLO laser-scanning, confocal microscope (Carl Zeiss Microscopy Gmblt, Jena, Germany) at a 20× objective. All experiments were conducted at room temperature and in the absence of

external electrical stimulation. Images were processed using ImageJ (v.1.49, NIH, http://imagej.nih.gov/ij).

Electrophysiology

For electrophysiological characterization of sinoatrial nodal cells, angiotensin II-treated CPCs were seeded onto gelatin-coated coverslips and allowed to attach overnight in CPC growth media. The action potentials of the sinoatrial nodal cells were then measured in current-clamp mode at a 5 KHz sampling rate (Axopatch 1D, Molecular Devices, San Jose, CA) and analyzed using AxoGraph. Borosilicate glass microelectrodes were used with tip resistances of 5–8 MΩ when filled with pipette solution. Spontaneous action potentials were recorded at 25°C using the perforated patch method. Briefly, 10 µL of 50 mg/ 50 µL amphoteracin B (Sigma-Aldrich, St. Louis, MO) in molecular biology grade DMSO (Corning, Corning, NY) was added to 1 mL of pipette solution, containing (mM): K-gluconate 130, Na-gluconate 10, NaCl 4, HEPES 10, GTP-Na·H_2O 0.3, and ATP-Mg·H_2O 4 (pH 7.4, adjusted with NaOH). The bath solution contained (mM): NaCl 136, KCl, 5, $MgCl_2 \cdot 6H_2O$ 1, $NaH_2PO_4 \cdot 2H_2O$ 0.3, $CaCl_2 \cdot 2H_2O$ 1.8, D-glucose 10, HEPES 10 (pH 7.3, adjusted with NaOH).

Epinephrine Dilution

To test the responsiveness of CPC-differentiated sinoatrial nodal cells to autonomic stimulation, bath solution was spiked to a working concentration of 5.5 µM epinephrine (Lot: BCBK3982V; Sigma-Aldrich, St. Louis, MO), as described in Taniguchi et al. (1981). Then, the membrane potential was recorded as described above.

Statistical Analysis

The Shapiro-Wilk test for normality was used to test the normality of data distribution. We then performed a two-tailed, paired t-test to compare the mean of all normally distributed data. Non-normally distributed data were compared using a Wilcoxon matched-pairs signed rank text. All data are reported as the mean ± the standard error of the mean. Prism 7 version 7.02 (GraphPad, La Jolla, CA) was used for all statistical analyses. P values < 0.05 were assumed to indicate statistical significance.

Results

Angiotensin II Receptor Type I Activation Induces Calcium Signaling Gene Expression

To activate calcium signaling in neonatal CPCs using angiotensin II, we first screened for the type 1 receptor, which functions via a G-protein coupled receptor to activate protein kinase C activation and calcium potentiation (Venema, 1998). Since the type 2 receptor is widely speculated to antagonize the type 1 receptor via heterodimerazation (AbdAlla et al., 2001), we also screened CPCs to select neonatal clones that primarily expressed the type 1 receptor (Figure 29A). Then, we treated cells expressing this receptor with angiotensin II for 72 hours and measured the induction of genes related to calcium signaling (Figure 29B): *PLCG1* (3.613 ± 0.008-fold change, $P<0.001$, n=3), *PRKCA* (7.493 ± 0.295-fold change, $P<0.001$, n=3), and *CAMK2A* (55.06 ± 9.986-fold change, $P<0.01$, n=3).

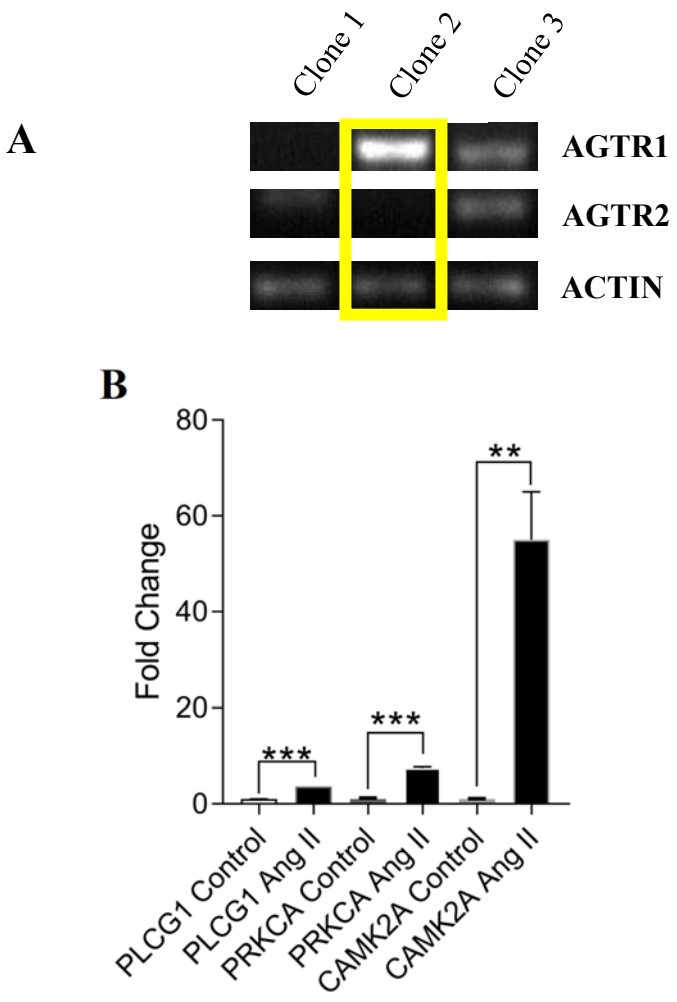


Figure 29. Neonatal cardiovascular progenitor cells expressing the type 1 angiotensin II receptor exhibit calcium activation following treatment with angiotensin II
Neonatal cardiovascular progenitors were screened for the expression of the type 1 angiotensin II receptor (AGTR1) using PCR and gel electrophoresis. The clone that expressed a higher ratio of the type 1 to type 2 (AGTR2) receptor (yellow box) was selected for further experiments (A). Then, CPCs with this receptor were treated with angiotensin II for 72 hours and calcium signaling gene expression was induced (B). n=3–6; **p<0.01, ***p<0.001. Changes in gene expression are shown as the mean ± S.E.M.

Treatment of Neonatal Cardiovascular Progenitor Cells with Angiotensin II Induces Expression of Sinoatrial Nodal Gene Program and Hcn4 Protein

Neonatal CPCs expressing the type I angiotensin II receptor were treated with angiotensin II for 72 hours. Changes in gene expression indicated the induction of the sinoatrial nodal gene program (Figure 30A): *TBX3* (2.613 ± 0.2781-fold change, P<0.01, n=3), *TBX5* (1.456 ± 0.224-fold change, P=0.152, n=3), *TBX18* (1.901 ± 0.025-fold change, P<0.01, n=3), *SHOX2* (2.273 ± 0.208-fold change, P<0.01, n=3), *HCN4* (4.135 ± 0.495-fold change, P<0.001, n=12),and *GJC1* (Cxn 45; 2.482 ± 0.256-fold change, P<0.05, n=3). Meanwhile, the cardiomyocyte gene program was repressed (Figure 30B): *NKX2-5* (0.197 ± 0.025-fold change, P<0.001, n=9), *TNNT2* (0.645 ± 0.087-fold change, P<0.05, n=3), and *GJA1* (Cxn 43; 2.482 ± 0.256-fold change, P<0.05, n=3). To confirm the induction of Hcn4 protein, which carries the funny current that is characteristic of pacemaker cells, we performed flow cytometry (Figure 30C) and western blot (Figure 30D). We observed a 7.75 ± 0.085-fold change in *Hcn4* protein expression (Figure 30E).

Angiotensin II-Treated Cardiovascular Progenitor Cells Exhibit Auto-rhythmic Calcium Flux

To test whether induced Hcn4 protein impacted the physiology of neonatal CPCs, we incubated treated cells with Fluo4-AM to detect calcium flux. We observed the generation of spontaneous auto-rhythmic calcium flux (Figure 31) in treated (black dots) CPCs. Such calcium activity was not observed in untreated (red dots) CPCs. (n=8 per group). Among treated CPCs, two trends emerged in spontaneous rhythm generation, as indicated by black and blue trend lines.

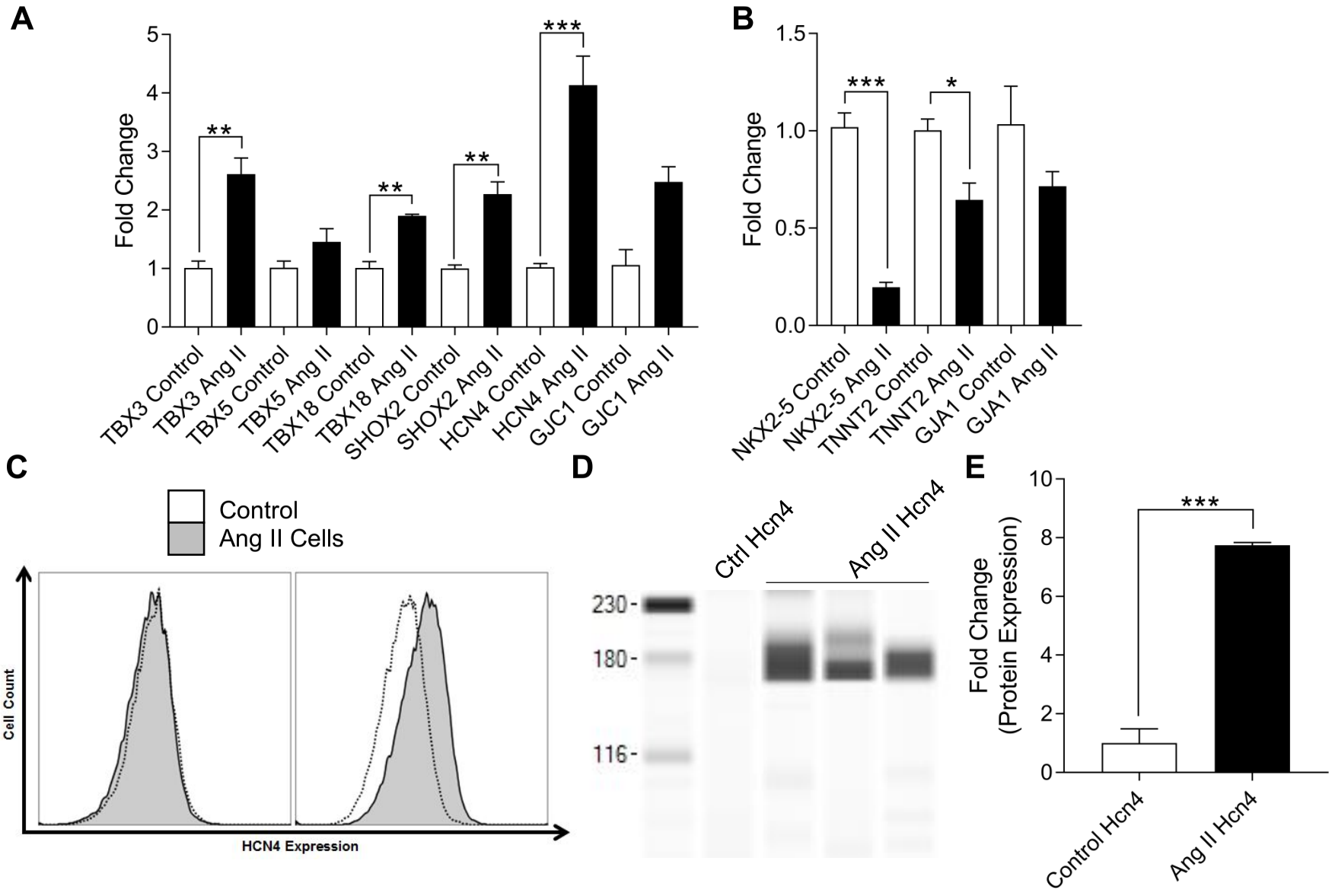

A
Fold Change
0
1
2
3
4
5
**
**
**
***
TBX3 Control
TBX3 Ang II
TBX5 Control
TBX5 Ang II
TBX18 Control
TBX18 Ang II
SHOX2 Control
SHOX2 Ang II
HCN4 Control
HCN4 Ang II
GJC1 Control
GJC1 Ang II
B
Fold Change
0.0
0.5
1.0
1.5
***
*
NKX2-5 Control
NKX2-5 Ang II
TNNT2 Control
TNNT2 Ang II
GJA1 Control
GJA1 Ang II
C
Control
Ang II Cells
Cell Count
HCN4 Expression
D
Ctrl Hcn4
Ang II Hcn4
230-
180-
116-
E
Fold Change
(Protein Expression)
0
2
4
6
8
10
***
Control Hcn4
Ang II Hcn4

Figure 30. Angiotensin II treatment induces the expression of genes and proteins characteristic of sinoatrial nodal cells
Neonatal cardiovascular progenitors were treated with angiotensin II for 72 hours. Then, the expression of genes involved in the sinoatrial nodal (A) and cardiomyocte (B) gene programs were measured by RT-PCR. Flow cytometry (C) and western blotting (D) was used to determine the fold-change in the expression of Hcn4 protein (E). n=3–12; *p<0.05, **p<0.01, ***p<0.001. Changes in gene and protein expression are shown as the mean ± S.E.M.

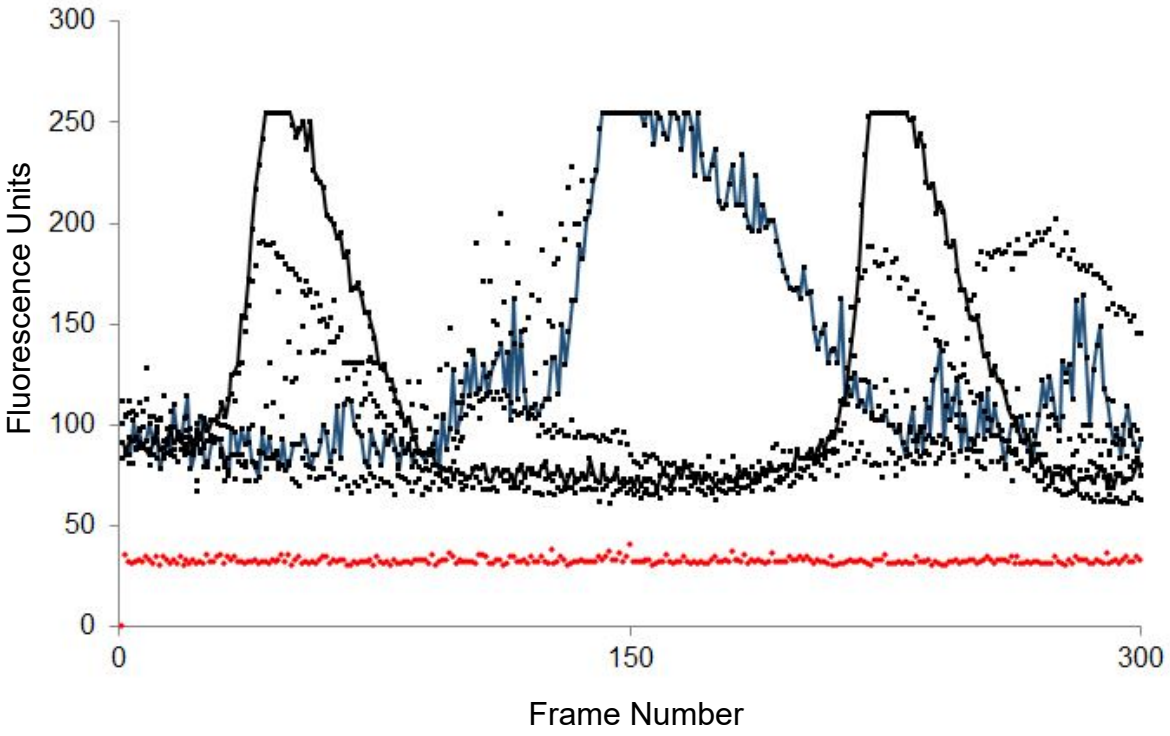
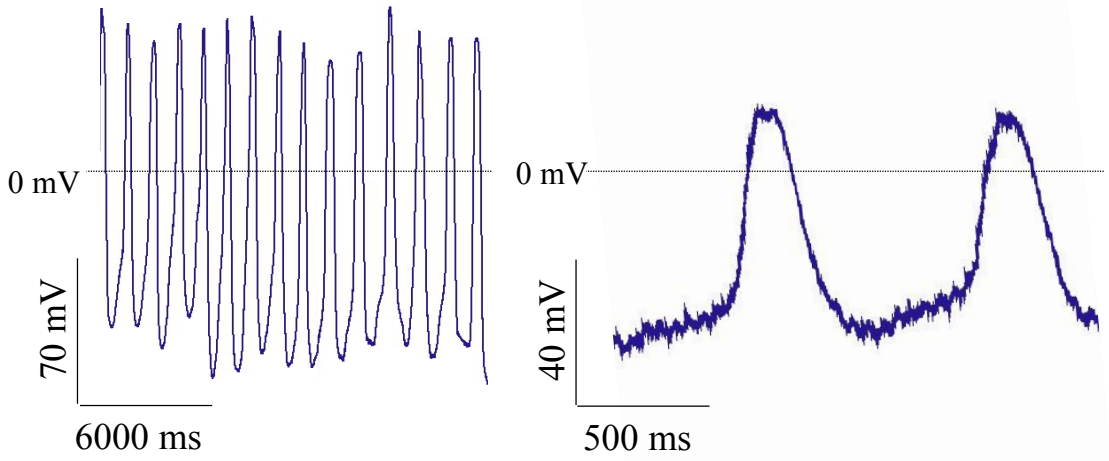


Figure 31. Angiotensin II treatment induces auto-rhythmic calcium flux
Neonatal cardiovascular progenitors were treated with angiotensin II for 72 hours, incubated with Fluor4-AM, and imaged using confocal microscopy. The intensity of calcium levels is indicated by the level of fluorescence. Black dots represent angiotensin II-treated CPCs, with black and blue lines representing the two observed calcium flux patterns. Red dots represent control CPCs. n=8 per group.

Angiotensin II-Treated Cardiovascular Progenitor Cells Have Functional Characteristics of Pacemaker Cells

Given that cardiomyocytes and various cardiac conduction system cell types (e.g., Purkinje fiber cells and atrioventricular node) exhibit auto-rhythmic currents, we performed whole cell electrophysiology recordings using the perforated patch method. Recording of angiotensin II-treated cells revealed an action potential of -40 ± 1 mV (n=9, Figure 32A), a slow maximum upstroke velocity (dV/dt_{max} < 10 V/s), and an epinephrine sensitivity that accelerated the number of action potentials from 1.05 ± 0.05 to 1.41 ± 0.05 per second (P<0.001, n=8; Figure 32B). Interestingly, the action potential duration at 50% of the maximum amplitude (APD50) of angiotensin II-treated CPCs was 289 ± 16 ms, which was relatively longer than the APD50 of SAN-like cells documented in Protze et al. (2017). This was possibly due to the difference in temperature at which recordings were performed (25°C versus 37°C). Notably, no sponteanously depolarizing membrane potential was observed in untreated CPCs



	Angiotensin II-treated Cells	SAN-like Cells Protze et al. (2017)
Action Potential Threshold	-40 ± 1 mV	-43 ± 3 mV
Maximum Upstroke Velocity	3.5 ± 0.4 V/s	≦ 10 V/s
APD50	289 ± 16 ms	65 ± 12 ms

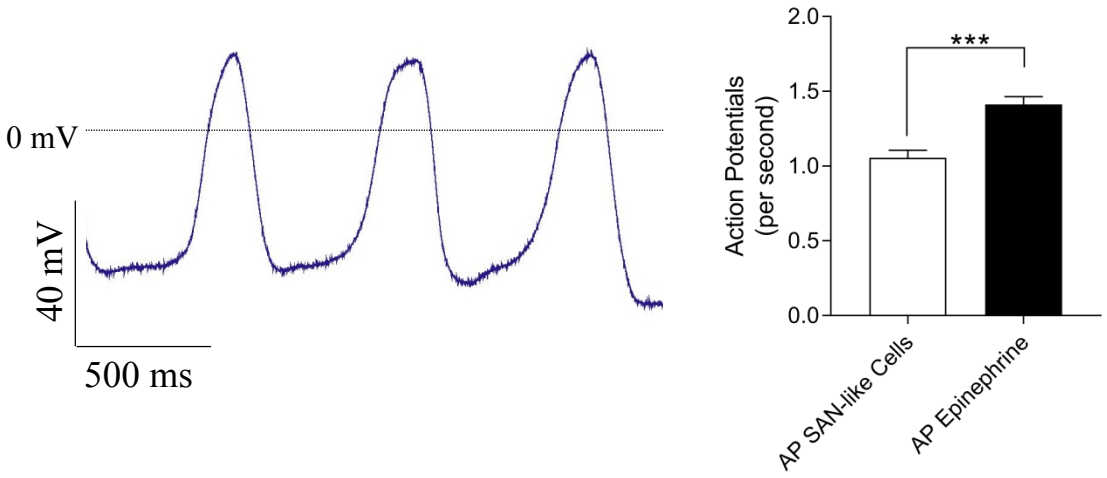


Figure 32. Pacemaker cell functional characteristics are induced in neonatal CPCs following angiotensin II treatment

The electrophysiology of neonatal cardiovascular progenitors that were treated with angiotensin II for 72 hours was recorded in the whole-cell, perforated-patch configuration. Representative membrane potential curves before and after filtering are shown as well as an analysis of action potential and maximum upstroke velocity (A). Epinephrine treatment reduced the time required to generate an action potential, as shown in a representative filtered membrane potential curve and by the increased number of action potentials per second (B). n=8–9; ***$p<0.001$. Change in the action potential count is shown as the mean ± S.E.M. AP, action potential; APD50, action potential duration at 50% of the maximum amplitude; SAN, sinoatrial node

Discussion

In activating calcium signaling using angiotensin II, we were able to reliably direct the induction of the sinoatrial nodal gene program, which correlated to elevated Hcn4 protein expression and auto rhythmic calcium flux. Using angiotensin II to activate calcium signaling follows from similar findings in our early population of neonatal cardiovascular progenitors that were treated with hWnt5a or exposed to simulated microgravity. This latter cell culture method has been shown to induce the sinoatrial nodal gene program in neonatal CPCs (Baio, 2018) and neural crest progenitor cells (Hatzistergos, 2018). Interestingly, non-canonical Wnt signaling, particularly Wnt11, is critically involved in the regulation of neural crest cell development and migration (De Calisto, 2005) along with the induction of CD166 (Gessert, 2006), which marks the precursor population of sinoatrial nodal cells (Scanone, 2013). Thus, the use of angiotensin II to induce non-canonical Wnt/Ca^{2+} signaling in early neonatal cardiovascular progenitor cells logically follows from the observed activation of calcium signaling under microgravity and the ability of non-canonical Wnt to enrich the sinoatrial nodal precursor population. These findings merit further research into the mechanisms governing the early events of sinoatrial node development, which will enable the ultimate construction of a biological pacemaker.

Recent work by the laboratories of Keller (Protze, 2017) and Foley (Brown, 2017) has produced important advances in the field of sinoatrial nodal development. Either overexpression of the TGFβ-activated kinase MAP3K7 (Brown, 2017) and Bmp4 titration at the onset of cardiogenic mesoderm induction (Protze, 2017) were used to create sinoatrial nodal-like cells. Interestingly, working cardiomyocytes, which exhibit

high contractility, exhibit a morphology that differs from that of sinoatrial nodal cells (Bakker, 2010). However, the enriched population of SAN-like cardiomyocytes generated by Protze et al. (2017) follows from observations in other laboratories that such cells can be produced in smaller percentages as a byproduct of inducing ventricular cardiomyocytes from ESCs using a standard protocol of temporally regulated moprhogens (Jha, 2016). Meanwhile, several working myocardial genes were found to be repressed in Map3k7-overepxressing ESCs, supporting the work of Brown et al. (2017) in generating sinoatrial nodal-like cells that are distinct from pacemaker-like cardiomycoutes. Future work is needed to clarify whether such cells are functionally capable of serving as a biological pacemaker over a prolonged period of time, or whether they will revert into a ventricular- or atrial-like cardiomyocyte upon transplantation. Nevertheless, the use of human-derived CPCs as seeds for the development of an *ex vivo* sinoatrial node is possible.

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CHAPTER FIVE

COMMENTS

In this dissertation, we presented findings of the effects of reduced gravity conditions on neonatal cardiovascular progenitor cells. In this concluding chapter, we seek to summarize some of the most salient features of our experiments in three areas: molecular changes borne by spaceflight, their Earth applications, and sinoatrial nodal development. Through the experiments described herein, we have been able to uncover features of the molecular response of early cardiovascular progenitor cells to reduced gravity conditions. Importantly, these findings contribute to the broad understanding of how humankind will fare in deep space and provide guidance for the expansion of our species beyond Earth. Moreover, features of the adaptive response exhibited by CPCs under simulated microgravity and aboard the ISS suggest augmented therapeutic potential, as indicated by enhanced proliferation, migration, and stemness marker expression. These features may continue to be explored and tested on Earth and in space in an effort to translate these findings into demonstrably effective stem cell-based cardiovascular regeneration and therapies. In this dissertation, we have already applied this strategy to the early stages of biological pacemaker development. Future work can extend these incipient experiments to expand the utility of CPCs in regenerative medicine on Earth.

As defined in chapter 2, the profound changes in developmental gene expression and enhanced proliferative potential experienced by CPCs during culture aboard the ISS are suggestive of augmented therapeutic potential on Earth. This prompted us to identify the molecular mechanisms by which CPCs adapt to reduced gravity conditions and to

understand how these effects could be recapitulated *in vitro* under normal gravity conditions. In doing so, we found that reduced mechanotransduction coupled with calcium signaling in a population of early cardiovascular progenitors can induce the expression of a modestly earlier developmental profile, as described in chapter 3. Finally, given that sinoatrial nodal cells are theorized to represent embryonic myocardium maintained in an undifferentiated state, we sought to understand whether the same signaling events that could induce an earlier developmental state under reduced gravity conditions could be applied to promote the development of pacemaker cells (chapter 4). When considered together, these studies advance our knowledge of the mechanisms governing the molecular adaptation to spaceflight and provide a plausible method for improving stem cell therapies and for inducing biological pacemaker cells.

Summary and Future Directions

The findings described in this dissertation provide novel insights into the molecular events underpinning cardiac stem cell function beyond the Earth-bound context. Yet, further research is needed to ensure the safe and effective application of these mechanistic findings in future clinical therapies. Moreover, the observations that simulated microgravity can result in the abnormal accumulation of pacemaker cells (Hatzistergos, 2018) along with an induction of the sinoatrial nodal gene program (Baio, 2018) merit further research into the incipient molecular events of the sinoatrial node's development. In these concluding remarks, we contextualize the findings described throughout this dissertation by discussing ongoing and future efforts to understand

cardiovascular development and how primordial cardiac cells can be used to facilitate regeneration following injury.

Reduced Gravity Conditions Impact Cardiogenesis

As humankind has expanded our presence in low Earth orbit and aims to undertake missions to deep space, organ system-wide changes have been observed within astronauts. In addition to bone loss, muscular atrophy, impairment of the immune system, and deformations in the ocular system, the cardiovascular system has been observed to undergo remodeling and reduced function in response to spaceflight (Frippiat, 2016; White, 2001). Researchers have moved to study these effects at a single cell level so as to develop approaches that improve the likelihood of human survival in deep space. As these studies have evolved, the potential use of microgravity in maintaining or enhancing the pluripotent capabilities of cells has benefited subsequent directed differentiation upon mechanical reloading.

A recent review of tissue engineering under microgravity conditions has demonstrated the increasing interest in leveraging the reduced gravity environment of various simulators or the ISS to prepare preliminary vessels, eye tissue, bone cartilage, and multicellular cancer spheroids (Grimm, 2018). Despite the apparent benefits of microgravity-inspired stem cell research, few studies have applied space research to cardiac progenitors or cardiac differentiation (Frippiat, 2016; White, 2001). Although several studies have successfully developed primitive vessels (Pietsch , 2017; Grimm, 2009; Grimm, 2010; Ma, 2014) from endothelial precursors (EA.hy926 or human cardiac microvascular endothelial cell types), only recently have researchers turned to studying

enhanced cardiomyocyte production (Jha, 2016) and augmented cardiac precursor stemness (Fuentes, 2015) under conditions of microgravity. Thus, our study of the effects of spaceflight on cardiovascular progenitors represents an important advance not only for cardiac adaptation and development in space, but also for identifying key mechanisms involved in the enhanced stemness that may represent a foundation for future methods of generating cardiac tissue.

In summary, we found that microgravity exerts broad effects on the developmental status, proliferative potential, and migratory ability of CPCs, some of which occurred in an age-dependent manner. In particular, neonatal CPCs exhibited increased expression of early developmental markers, enhanced proliferative potential, and increased migratory capacity following spaceflight. These findings suggest that broad cytoskeletal modifications resulting from reduced mechanotransduction impart improved migratory and adhesion capabilities and that CPCs can propagate or experience additional intracellular signaling events (e.g., calcium signaling) that modify their developmental status. Until now, most studies of cardiovascular adaptation to spaceflight have focused on terminal cardiomyocytes or organ system-wide alterations (Hughson, 1985; Blue, 2015; Hughson, 2017; Negishi, 2017). Conversely, studies of stem cells have focused largely on embryonic or induced pluripotent stem cells (Blaber, 2015; Shinde, 2016; Jha, 2016). Thus, our findings provide unique information on the effects of spaceflight on cardiac lineage development. In the context of cardiovascular stem cell therapy, these results represent one avenue by which to procure a cardiac-committed stem cell with enhanced regenerative properties. This follows from increasingly strong evidence that supports the use of progenitors that are only nascent in their commitment to the

cardiovascular lineage in promoting cardiovascular repair. Thus, research in cardiovascular regenerative medicine must also consider methods that enhance the regenerative potential of CPCs.

In a study of repair following myocardial infarction in non-human primates, Blin et al. (2010) studied the efficacy of an early-stage cardiovascular progenitor that expressed stage-specific embryonic antigen 1 (SSEA-1), mesoderm posterior 1 (MESP1), and OCT4. In doing so, the researchers observed differentiation of these early cells into ventricular cardiomyocytes, thereby healing approximately 20% of the scar tissue. The use of such a progenitor was demonstrated to be efficacious in a rodent model of myocardial repair (Bellamy, 2015). Meanwhile, Menasché et al. (2015) reported the first clinical case report of treatment using this early cardiovascular progenitor cell type. In brief, transplantation of SSEA-1- and Islet1-expressing CPCs derived from embryonic stem cells resulted in improved cardiovascular function. As shown in Table 2, studies of stem cell-based cardiovascular repair have revealed an emerging pattern in which an earlier cardiovascular progenitor more effectively improves cardiovascular function.

As future research into cardiovascular progenitor adaptation to spaceflight evolves, key questions must be answered. First, what are the most critical signaling components that account for the CPC response to spaceflight and subsequently enhanced stemness? Second, can these effects be recapitulated *in vitro* on Earth in the absence of simulated microgravity or ISS-borne laboratories? Although these questions have been addressed, at least in part, through our work in this dissertation, further questions persist. What is the optimal "dose" of microgravity for stem cell-based repair and, as a corollary, does low Earth orbit (e.g., the ISS) and deep space (e.g., a mission to Mars) produce the

same effects? Finally, what implications do these findings have for cardiac development in the context of extraterrestrial conception and *in utero* development? We are only beginning to uncover the wide-ranging implications of spaceflight for human biology.

Signaling Events Involved in CPC Adaptation to Spaceflight Can Be Applied on Earth

As described above, the molecular mechanisms underpinning the adaptation of CPCs to spaceflight and simulated microgravity will facilitate the enhanced use of these cell types in ground-based therapies. Thus, we worked to identify the involvement of key signaling pathways in this process. As summarized below, the involvement of calcium and Akt signaling in primordial cardiovascular cells are critical to this process.

In the context of cardiogenesis, studies performed in both embryos and ESC-derived cardiomyocytes have shown a critical role of Ca^{2+} in regulating multiple steps of heart formation (Pucéat, 2005). For example, induction of Ca^{2+} oscillation promotes proliferation and, upon transplantation, enhances engraftment and expansion (Ferreira-Martins, 2009). In this way, our observed shift in expression of markers of early cardiac development is likely the result, at least in part, of modified calcium signaling activity within neonatal CPCs. Furthermore, research into Mesp1-expressing pre-cardiac mesoderm derived from human ESCs identified enriched activity along the calcium, extracellular matrix-receptor, and Wnt signaling pathways at day 5 of development (den Hartogh, 2016), which is supported by our observed increase in markers of such a state of development after culture aboard the ISS (chapter two) and using SMG (Baio, 2018).

Interestingly, the ability of spaceflight to impact epigenetic regulation is only in its nascent stages of study, but, when further understood, may provide insights into the mechanisms governing enhanced differentiation potential under conditions of microgravity. In human lymphoblastoid cells, DNA methylation patterns were found to be widely altered by simulated microgravity (Chowdhury et al, 2016), with Singh et al. (2010) reporting that simulated microgravity preferentially induces hypomethylation in human lymphocytes. It could be speculated that microgravity can induce hypomethylation in cardiovascular progenitor cells, and further study in this area represents one interesting avenue for future research. As demonstrated in this dissertation, cardiomyocytes were induced from CPCs using treatment with 5-azacytidine, which inhibits DNA methyltransferase and results in near global hypomethylation. While the exact mechanism of this treatment is unknown, Notch-related signaling components, which promote cardiomyocte induction by inhibiting canonical Wnt signaling during the early stages of cardiogenesis (Gessert & Kühl, 2010), remain uniquely hypomethylated following removal of 5-azacytidine from cell culture (Ramos et al., 2015) suggesting that hypomethylation events are important to differentiation processes. Thus, the role of microgravity-induced alterations in epigenetic control of stem cells, and of methylation events in particular, merits further study.

In addition to identifying the role of calcium signaling in mediating some of the changes experienced by neonatal CPCs, we also found that spaceflight impacted adult CPCs in a manner that may not yet be fully appreciated. Therefore, the mechanisms of CPC biology appear to be age-dependent. The findings outlined in this dissertation can be used to guide future experiments that characterize such changes and ultimately develop

therapies that can enable the use of adult-derived CPCs in an autologous manner. Recent work in our own laboratory (Fuentes, 2013) has identified functional differences in signaling pathways pertinent to many of the processes observed under simulated microgravity. Similarly, the secretome of adult and neonatal CPCs were recently compared using liquid chromatography tandem mass spectrometry (Sharma, 2017). In this study, researchers identified elevated protein expression in pathways related to stem cells maintenance, differentiation, heat shock proteins (i.e., stress response), proliferation/growth, anti-apoptosis, and calcium regulation in tissue culture media of neonatal CPCs. Conversely, tissue culture media of adult CPCs was found to be enriched in proteins related to aging, senescence, and anti-proliferation.

This prompts an important line of inquiry to understand the molecular basis for functional differences between adult- and neonate-derived cardiovascular progenitor cells. While we have shown here that CPCs derived from both age populations can similarly express developmental markers and exhibit an ability to differentiate (though to differing extents), their functional differences in response to various conditions, including simulated microgravity (Fuentes, 2015), spaceflight, hypoxia (Hernandez, 2018), and myocardial infarction (Sharma, 2017), indicate fundamental differences in the mechanisms governing age-dependent CPC function.

Notably, both Sharma et al. (2017) and Hernandez et al. (2018) detected a difference in the induction of heat shock proteins between neonatal and adult CPCs. Moreover, the former research group demonstrated that heat shock factor 1 is an upstream regulator of many of the pathways uniquely enriched in the neonatal CPC secretome. Thus, the age-dependent response of CPCs to various conditions, including

spaceflight, may be a reflection of the age-dependent readiness of cells to mount a stress response and induce down-stream effects. Experiments that assess the delayed response of adult CPCs to spaceflight may refine this hypothesis and provide a plausible path for enhancing the utilization of adult patient-derived cells. This is particularly important given that adult patients would more regularly require regenerative therapies for the heart.

Ultimately, future research will continue to uncover the utility of microgravity-treated CPCs in cardiovascular repair. Our observed enhancements in migration and proliferation along with the induction of an earlier developmental state compliment the emerging trend of early cardiac progenitor cell transplantation. As we have elucidated the involvement of key signaling pathways in the process of CPC adaptation to spaceflight, we can begin to leverage the observed enhanced therapeutic potential of spaceflown or simulated microgravity-exposed cardiac progenitors on Earth. As shown in this dissertation, one unique application of such findings is the development of an *in vitro* biological pacemaker.

The Sinoatrial Node Can Be Induced in Early Neonatal Cardiovascular Progenitor Cells

Some astronauts experience arrhythmias during spaceflight (Anzai, 2014). The mechanisms by which these irregularities in heart rhythm develop are unknown, but researchers speculate that diet imbalance and neurohormonal dysregulation are possible causes. Among the latter, regulators of heart rhythm, such as angiotensin II (Delpón, 2005; Watenpaugh, 2001; Xu, 2009), are differentially regulated by spaceflight and merit

further attention. Reduced gravity conditions have also been implicated in the dysregulation of myocardial and sinoatrial nodal gene program expression in endogenous progenitors. Indeed, simulated microgravity has been shown to induce the sinoatrial nodal gene program in neonatal CPCs (Baio, 2018) and neural crest progenitor cells (Hatzistergos, 2018). Such alterations to cardiac pacing and progenitor developmental gene expression under reduced gravity prompted us to further explore the means by which signaling events under these conditions may promote sinoatrial nodal development. Interestingly, non-canonical Wnt signaling, particularly Wnt11, is critically involved in the regulation of neural crest cell development and migration (De Calisto, 2005) along with the induction of CD166 (Gessert, 2006), which marks the precursor population of sinoatrial nodal cells (Scanone, 2013). Thus, the use of angiotensin II to induce non-canonical Wnt/Ca^{2+} signaling in early neonatal cardiovascular progenitor cells logically follows from the observed activation of calcium signaling under microgravity and the ability of non-canonical Wnt to enrich the sinoatrial nodal precursor population. Furthermore, the induction of *TGFB1* expression under SMG (Baio, 2018) also supports the observation that Tgfβ-activated kinase signaling in embryonic stem cells faithfully induces functional sinoatrial nodal cells (Brown, 2017).

When considered together, this evidence suggests that sinoatrial nodal induction requires a sufficiently developmentally early cell type. This is supported by observations by Bressan et al. (2013), who traced sinoatrial nodal development in an avian model and found that the SAN developed from a tertiary heart field that did not express Nkx2-5 or Isl1. Importantly, early mesodermal cues were able to promote the development of this specialized region shortly after gastrulation, prior to the onset of cardiac morphogenesis.

This is reflected by the nearly unanimous use of embryonic or induced pluripotent stem cells in engineering biological pacemaker cells. Thus, our findings that angiotensin II treatment can induce the sinoatrial nodal gene program and elicit a sinoatrial nodal-like phenotype in CPCs provides plausible evidence that a sufficiently developmentally early cardiovascular progenitor may be suitable for pacemaker cell induction.

Importantly, further work must be performed to determine if angiotensin II-treated cells can retain a SAN-like phenotype beyond the period of treatment and, ultimately, following transplantation and integration into the host myocardium. Other laboratories have induced a sinoatrial nodal-like phenotype by transforming ventricular myocytes in a pig and guinea pig with *TBX18* transfection using an adenoviral vector (Hu et al., 2014; Kapoor et al, 2013). In these studies, researchers observed a decline in pacemaking efficacy approximately two weeks after injection, which was speculated to be the result of immune-mediated clearance of the adenovirus. Meanwhile, in fetal mice, *TBX18* was found to be insufficient to induce SAN differentiation of chamber cardiomyocytes, thereby suggesting that the transcription factor may be relevant to sinoatrial nodal development, but not specific to its fate nor sufficient for its induction (Greulich et al., 2016). This complicates our ability to accept the reliability of *TBX18* induction as being sufficient for sustained sinoatrial nodal reprogramming. Furthermore, recent work from the Keller group (Protze et al., 2017) demonstrated that functional SAN induction and subsequent integration following transplantation into rodents is possible, but similarly only assessed the *in vivo* efficacy after two weeks. Thus, longer-term studies (i.e., > 2 weeks) are needed to ensure that induced sinoatrial nodal cells are able to maintain their phenotype, successfully interdigitate with the surrounding working

myocardium, and serve as a reliable and non-arrhythmogenic pacemaker following transplantation.

Meanwhile, work by the Foley group (Brown et al., 2017) demonstrated an important role of Tgfβ signaling early in development, which resulted in the induction of *HCN4* within days of embryoid body formation. Interestingly, this follows from the identification of a distinct and temporally early population of mesoderm that gives rise to the sinoatrial node (Bressan et al., 2013). Furthermore, our finding that 72 hours of treatment with angiotensin II was sufficient to induce a pacemaker-like phenotype in a CPC population, as described in chapter four, overlaps with the timeline of *HCN4* induction in Brown et al. (2017). While Protze et al. (2017) and the *TBX18* transfection studies either successfully reprogramed myocytes or induced cardiomyocytes into cells with pacing potential, these derivatives still retain features of cardiomyocytes and may therefore be susceptible to the extracellular environment of the surrounding myocardium following transplantation, which may revert sinoatrial nodal-like cardiomyocytes back into working cardiomyocytes. Although neither the Foley group nor our laboratory have tested SAN-like cells *in vivo*, doing so would be critical to test this hypothesis as both groups have prepared pacemaker-like cells that exhibit repression of working myocardial genes and may thus be able to sustain the pacemake phenotype beyond two weeks.

Ultimately, these findings merit further research into the mechanisms governing the early events of sinoatrial node development, which will enable the ultimate construction of a biological pacemaker. Furthermore, the observed relationship between simulated microgravity/spaceflight and sinoatrial nodal gene program/pacemaker cell

accumulation indicate that pre-cardiovascular developmental stages, such as the incipient mesodermal or mesendodermal stages, merit further research.

Conclusions

As humans prepare to expand our presence in space, it is imperative to deepen our understanding of the nature of cellular adaptation to reduced gravity so that we may develop mechanisms by which these molecular changes in cardiac cell types can be countered. As experiments into the mechanisms governing human development and function in spaceflight evolve, it will be imperative to continue to apply these findings to regenerative therapies and tissue engineering. Ultimately, further exploration of the therapeutic potential of space-flown cardiovascular progenitor cells may one day yield substantial benefits for cardiac repair on Earth.

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